The Thesis committee for Jon Peter Gengler

Certifies that this is the approved version of the following thesis:

Biosynthesis of Sulfur Containing Heterocycles in Natural Products

Approved by

Supervising Committee

Supervisor Hung-wen Liu

Walter Fast

Biosynthesis of Sulfur Containing Heterocycles in Natural Products

by

Jon Peter Gengler,B.S.

Thesis

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Dedicated to my family, especially my parents, Dan and Lucy Gengler, who always believed in me far more than I believed in myself

Biosynthesis of Sulfur Containing Heterocycles in Natural Products

by

Jon Peter Gengler,M.A.

The University of Texas at Austin,2014

Supervisor: Hung-wen (Ben) Liu

This thesis is a comprehensive review of the biosynthesis of sulfur containing heterocycles in natural metabolites. The review focuses on sulfur incorporation and cyclization of the moieties, with a lesser examination of the role these heterocycles play in the chemistry of their compound's activity.

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1 Introduction

The potential of synthetic biology is difficult to overstate. To be able to design biosynthetic pathways to produce complex molecules cheaper, more safely, and more environmentally friendly than current synthetic methods could open vast possibilities both in science and industry. Unfortunately, much of this potential is little more than an exciting dream, with concrete applications limited in scope and lacking economic competitiveness, but this could be said of any nascent field in its early days. One of the deficits in this field is the lack of knowledge about how specific moieties are attached to their natural products, restricting the field to protein systems that nature has already made modular and inhibiting efforts toward more diverse structures. The purpose of this review is to examine one broad type of moiety for which information is relatively lacking, sulfur containing heterocycles. By highlighting what we know and what we don't about this diverse range of compounds, this review aims to serve synthetic biologists by informing them what tools are at their disposal, as well as inform enzymologists and others where there is opportunity to expand our knowledge in this area.

The review itself is broken into eight broad categories based on the structure of the moieties discussed and then further subdivided into categories based around classes of compounds when applicable and individual cases when not. A complete examination of each compound's biosynthesis would require far more than a single review and therefore this review is focused on the incorporation of sulfur into the compounds and the formation of the heterocycle. An effort has also been made to mention those compounds that are likely to be produced through relatively novel biological mechanisms, but have a deficiency of information on them.

2 Disulfide Heterocycles

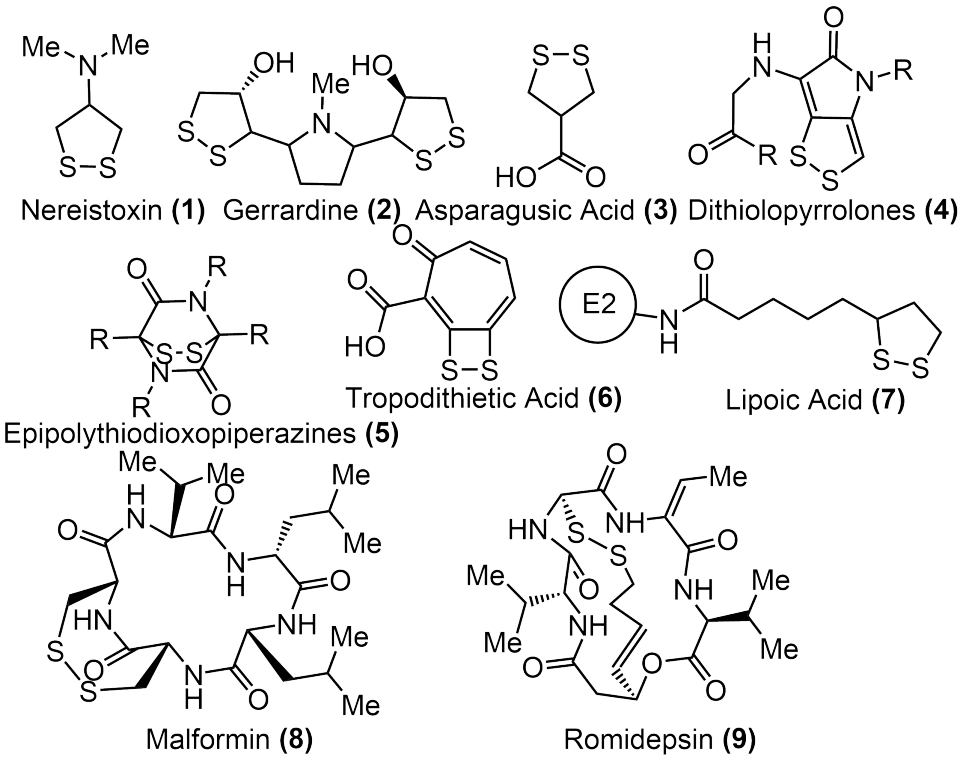

Figure 1: Metabolites with heterocycles containing a disulfide bond.

With bond energies on the order of 250 kJ/mol, disulfide bonds are significantly less stable than the carbon carbon bonds that make up the bulk of biological molecules.[1] However, this bond is sufficient to stabilize compounds at normal temperatures for most cellular environments, so long as they are in an oxidative environment. Disulfide bond formation is metabolically favorable for the cell to make often producing reducing equivalents that can be used in ATP generation, or at most consuming ambient oxygen.[2] Because of this disulfide bonds are a common motif in protein tertiary and quaternary structures, especially in excreted products.

While it is possible for a wide variety of cofactors to oxidize thiols in the formation of a disulfide bond, the most common oxidant used in vivo appears to be another disulfide bond formed within the thioredoxin motif CxxC,[2,3] found on

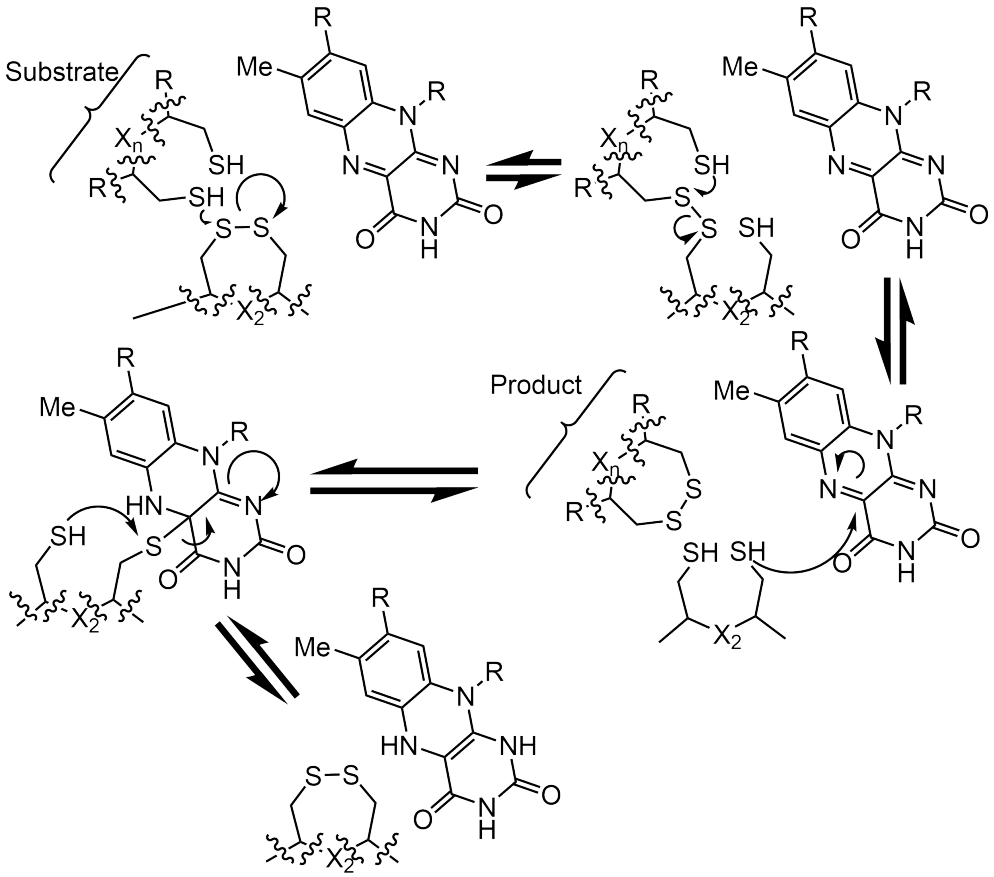

Figure 2: General disulfide forming reaction.

an alpha helix positioned proximal to a flavin binding site. As shown in figure 2, this placement allows the CxxC disulfide to be oxidized by the flavin, which then transfers the two electrons either to NAD+ or O_2 to regenerate the oxidized enzyme for another round of catalysis. Four clades of disulfide forming enzymes have been identified with sequence similarities consistent with the type of substrates they oxidize, peptide oxidases like TrxR, epidithiodioxopiperazine **5** oxidases like GliT, dithiolopyrrolone **4** oxidases like HlmI, and DepH like oxidases.[4–6]

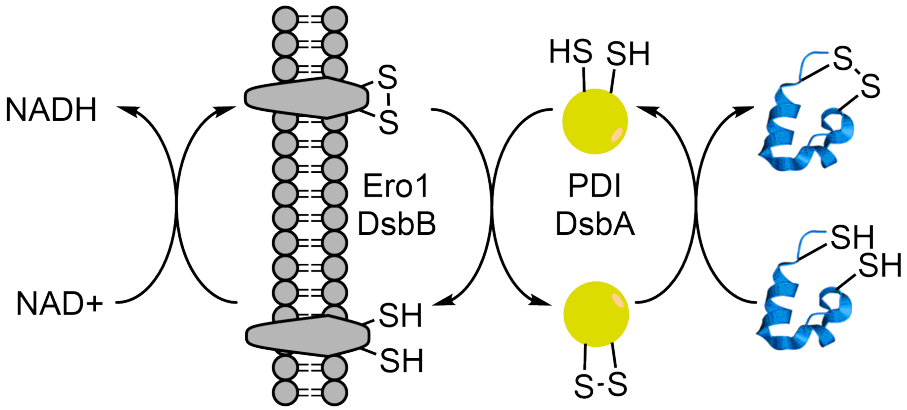

Figure 3: Generalized peptide disulfide oxidizing system.

2.1 Peptide Disulfides

Disulfide bonds are often used in secreted enzymes to stabilize their structure. Ion concentration, pH, and other conditions that determine a protein's conformation will vary outside the protective membrane of a cell, but it can reasonably be expected that the environment will be oxidative enough to maintain a stable disulfide bond. As disulfide bonds are used to stabilize large protein structures, it is unsurprising that they are also used to form macrocycles in many secondary metabolites, such as conotoxins, thionins, defensins, cyclotides and other short polypeptides.[7–11] Of these macrocyclic compounds, the most structural research has been done on conotoxins.

No specific thioredoxin has been identified for any of the aforementioned peptide products, each of which appear to use the same promiscuous oxidase as larger protein disulfides. The most well studied systems are Ero1 and PDI in eukaryotes, and the Dsb family of proteins in prokaryotes. Other peptide disulfide oxidation systems have been identified,[12] but no evidence has been published to suggest that these alternitive enzymes play a significant role in the oxidation of peptide secondary metabolites. As shown in Figure 3, these enzymes appear to work in pairs with the soluble protein, PDI or DsbA, acting in the endoplasmic lumen or the periplasmic space to oxidize the target. A membrane associated

protein, Ero1 or DsbB, then reoxidizes the soluble protein and shuttles electrons to an electron acceptor.[2]

As many of these metabolites have multiple thiols able to form disulfide bonds, it is reasonable to expect that a promiscuous oxidase could catalyze improper bond formation. Experiments involving rapid quench, limited oxidation agent concentrations, and conditions that destabilize the substrate's structure confirm that improper disulfide bonds do form.[10,13] However, improper disulfide bridging is rare in fully oxidized products.[10,11,13] It has been hypothesized that partially oxidized products form an equilibrium between all possible bridges, with formed bonds oxidizing reduced thiols in a series of intramolecular reactions.[13] Proper bond formation appears to be cooperative,[13,14] which is indicative of thermodynamic control of proper bridging, since the most stable bridging pattern will bring properly interacting sulfurs closer together, allowing for easier oxidation by another oxidizing equivalent.[10,14] Still, there do appear to be kinetic traps for numerous peptide products, and it has been shown that PDI may help peptides escape these traps by reducing improper bonds under certain conditions and allowing the peptide to fold into a more stable confirmation.[2,10,11,14]

2.2 Epipolythiodioxopiperazines

The epipolythiodioxopiperazine **5** familly of compounds includes over a dozen metabolites each containing a piperazinedione ring bridged by a disulfide bond. The toxisity of this class of compounds seems to come predominantly from its reduced form catalyzing the formation of reactive oxygen species (ROS).[15–17] Epipolythiodioxopiperazines can also form covalent adducts with proteins,[15–19] though the fact that this would involve the less toxic oxidized form of the compound,[5,15–17] and the lack of specific protein targets makes it unclear how significant these adducts are for toxicity. The formation of the disulfide ring by the producing organisms appears to serve two purposes. First it is protective,

preventing the toxic compound from damaging the cell.[20] Secondly it appears to increase the lipophilicity of the compound. After leaving the producing cell the disulfide is maintained by the surrounding environment so that it may enter the target cells. Once inside the more reducing environment of the cytoplasm, the epipolythiodioxopiperazine's disulfide bond is reduced, making the compound more hydrophilic. Unable to diffuse out of the cell after being reduced, epipolythiodioxopiperazines accumulate in the target cells, enhancing the compound's toxicity.[20,21] Paradoxically, a secondary function of epipolythiodioxopiperazines **5** may be to protect cells from exogenous free radicals, as demonstrated in *A. fumigatus* strains producing the epipolythiodioxopiperazines **5** compound gliotoxin. *A. fumigatus* increased gliotoxin production in the presence of hydrogen peroxide and gliotoxin mutants showed greater susceptibility to hydrogen peroxide than the wildtype or mutants with gliotoxin added exogenously.[22]

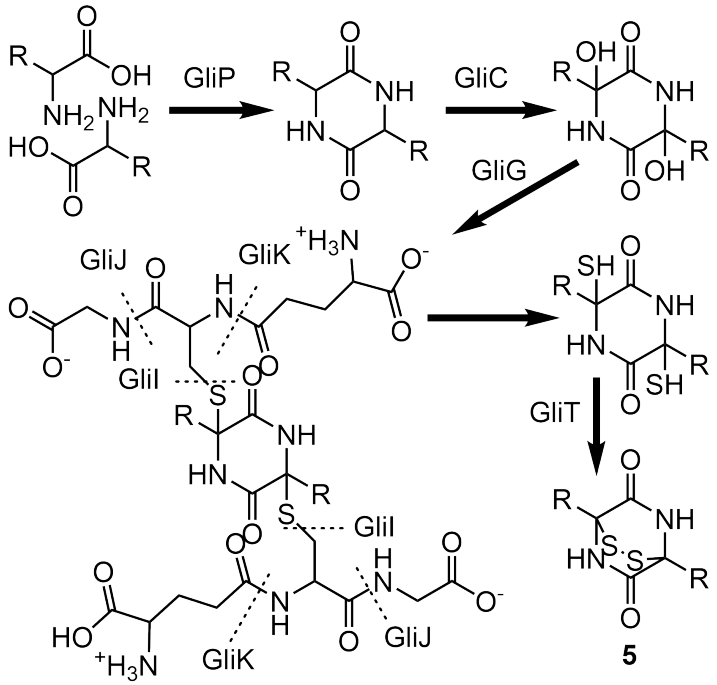

Figure 4: Epipolythiodioxopiperazines core formation.

While recent work has advanced our understanding of many epipolythiodioxopiperazines' **(5)** biosynthesis,[23–26] by far the most studied case is the biosynthesis of gliotoxin (Fig 4), the first discovered epipolythiodioxopiperazine **5** product.[27] Epipolythiodioxopiperazines **5** exhibit a moderate degree of diversity in their structures, but the core bicyclic rings are highly similar. It is therefore likely that the biosynthesis of this core is conserved within this class of compounds. In gliotoxin the piperazinedione ring **5** is formed by a pair of coupling reactions catalyzed by GliP, a nonribosomal peptide synthase (NRPS), between phenylalanine and serine to form a cyclic dipeptide.[16,22,27] The sulfur incorporation proceeds first by the activation of what was once the two alpha carbons by a hydroxyl group, catalyzed by the monooxygenase GliC.[22,28] This allows for the nucleophilic attack by glutathione, catalyzed by GliG, to form the C-S bonds.[16,27,29] GliK and GliJ then sequentially remove glutamate and glycine from the glutathione appendage, respectively.[30] The final cleavage of the remaining cysteine is performed by GliI in a PLP-dependent reaction which produces ammonium and pyruvate, and leaves a free thiol attached to the piperazinedione ring.[31]

These thiols are oxidized by GliT, an FAD-dependent oxygenase that shares a number of similarities to the thioredoxin family involved in peptide maturation.[16,22] A pair of cysteines shuttle the electrons to an FAD where they can then be transferred to dioxygen (Fig 2).[5,27] The use of oxygen instead of NAD(P) as the final oxidant may be more energetically wasteful, but it drives the reaction forward to minimize the quantity of potentially harmful reduced gliotoxin in the cell.

2.3 Dithiolopyrrolone

The dithiolopyrrolone **4** family of antibiotics is defined by a bicyclic heterocycle skeleton consisting of a pyrrolone ring decorated by an amino group, which is fused

with a five-member disulfide containing ring.[32] The family can be further subdivided into holomycin type antibiotics if the pyrrolone amine group is methylated, and thiolutin type antibiotics if it is not. For holomycin compounds this methyl group appears to be attached after the dithiolopyrrolone ring **10** is formed, as shown in Figure 5.[33] Beyond this separation, further diversity is acheived by the attachment of various acyl groups to the amino group.[32,34–37] One particularly notable variant is thiomarinol, which attaches the biologically active marinolic acid to the amino group to form a hybrid antibiotic with higher activity than either compound individually.[38,39]

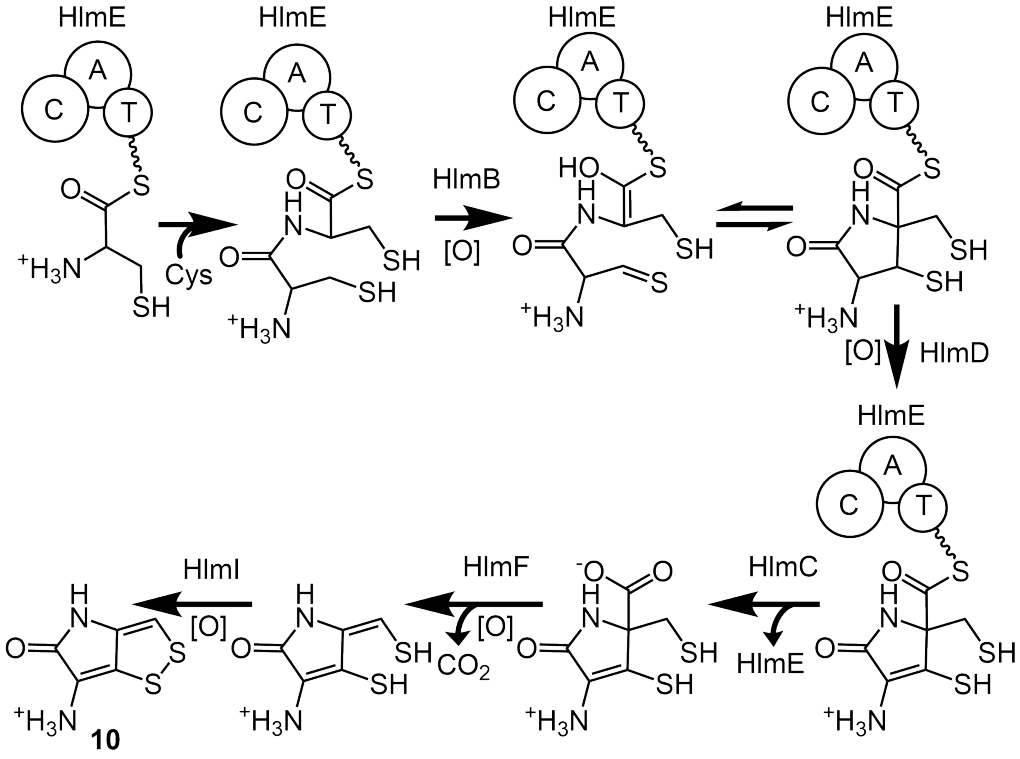

Figure 5: Formation of the holothin dithiolopyrrolone core **10**.

The enzymes shown in Figure 5 are from the holomycin biosynthetic pathway. Homologs for the hlm cluster are found in all characterized dithiolopyrrolone gene clusters. Hence, it is generally accepted that the dithiolopyrrolone ring **10** is formed by the same mechanism in other

pathways.[33,36,38] The initial enzyme in forming the holothin ring **10** is HlmE, an NRPS protein, which catalyzes the condensation of two cysteine molecules into a dipeptide and then cyclizes the pyrrolone ring with help from the flavin dependent acyl-CoA dehydrogenase HlmB. HlmB catalyzes the formation of a double bond between the sulfur and the β-carbon of one cysteine to allow for nucleophilic attack by the α-anion of the other cysteine.[36] The formation of the dithiol bond to close the second ring is catalyzed by HlmI. The active site of this protein is highly similar to other disulfide forming enzymes, with two cysteines on an alpha helix shuttling electrons to an FAD which then transfers them to a soluble electron acceptor (Fig 2). Like GliT this electron acceptor appears to be oxygen, driving the reaction in favor of disulfide formation.[4,32,35,40]

Dithiolopyrrolone antibiotics have been demonstrated to act by inhibiting transcription, possibly by forming disulfide adducts with target proteins.[32,35,40] The formation of the bicycle through oxidation of the disulfide bond appears to be predominantly a resistance mechanism for the producing cell. Organisms lacking HlmI exhibit significantly greater susceptibility,[4] unless they possess an alternative resistance gene such as an RNA methyltransferase found in *Yersinia ruckeri*.[32,40] This strongly suggests that, like gliotoxin, holothin requires a reducing environment to be active. The attachment of an acetate side chains to structure **4** accelerates non-enzymatic oxidation of the disulfide bond.[4] This modification probably helps to maintain the more lipophilic oxidized form of the compound in the extracellular environment so, like gliotoxin, the compound can diffuse into the target cells and accumulate in the more reductive environment.

2.4 Romidepsin-Like Compounds

Romidepsin (FK228) **9**, thailandepsin, FR-901228, FR-901375 **11**, and spiruchostatin (burkholdac) **12** are all histone deacetylase (HDAC) inhibitors of similar structures and, presumably, similar biosynthetic mechanisms. Their

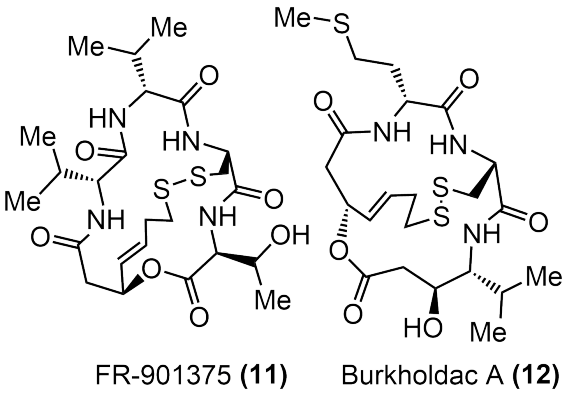

Figure 6: Example romidepsin-like compounds.

structures can be split into two primary components, a cyclic depsipeptide and a hydrophobic four carbon tail ending in a thiol. This terminal thiol group is derived from a cysteine, which is the starting unit for a PKS system responsible for building the hydrophobic tail. This is then fed into an NRPS complex which catalyzes the formation of the bulk of the compound. Which specific amino acids are incorporated and whether an additional PKS system is used in the depsipeptide moiety depends on the compound, but a cysteine is incorporated early, either as the second or the third amino acid to be attached in the growing peptide chain. This peptide chain is then cyclized to form the depsipeptide. This is followed by disulfide bond formation between the two cysteine thiols to complete the assembly of the bicyclic compound.[41–44]

Romidepsin **9** can be oxidized nonenzymatically by ambient oxygen,[6] though all identified biosynthetic clusters also contain an oxidase to accelerate the process.[43–45] The enzyme responsible for this final oxidation, DepH for romidepsin **9**, is also similar in mechanism to GliT and HlmI, with a pair of cysteines transferring electrons to an FAD and ultimately an oxygen (Fig 2).[6,41,44,46] The binding site for the oxygen has some of the features of an NAD(P) binding site, but several amino acids appear to have been mutated to prevent NAD(P) binding to this site. Notably Glu138, Ala197, Ser198 and Asp202, all hydrophobic or negatively charged amino acids, occupy spaces that would be occupied by

positively charged residues in NAD(P) binding proteins.[46]

The purpose of the formation of the bicyclic moiety is less clear in romidepsin related compounds than it is in dithiolopyrrolones **4** and epipolythiodioxopiperazines **5**. The reduced form of romidepsin **9** has been proposed to act by chelating the active site zinc ion of HDAC1 and HDAC2 using the thiol at the end of the tail, with the depsipeptide cycle interacting with the surface of the protein to provide a target specific plug for the channel.[42,47,48] It is also has been found that reduced romidepsin **9** can inhibit PI3K by binding to its ATP binding pocket.[48] However, the oxidized form has also been found to be more active against HDAC3 than the reduced form.[42] The negligible antibacterial activity of romidepsin **9**[49] makes it unlikely that there would be much pressure to oxidize the compound as part of a resistance mechanism. It is likely that similar to dithiolopyrrolones **4** and epipolythiodioxopiperazines **5**, the oxidized form is better able to accumulate in target cells, though there is no direct evidence to support this hypothesis.

2.5 Lipoic Acid

Lipoic acid **7** is a cofactor vital to the activity of α-ketoglutarate dehydrogenase (Fig 7), pyruvate dehydrogenase (Fig 25), branched chain oxo-acid dehydrogenase, acetoin dehydrogenase, and glycine cleavage.[50] It consists of octanoic acid with sulfurs attached to carbons six and eight. The active cofactor is bound to lipolated proteins through a peptide bond to a lysine residue on the target protein.[51] The cofactor alternates between a cyclic oxidized form, which allows it to undergo an electrophilic attack by the newly decarboxylated α-anion equivelent, and a reduced form after transferring the substrate to an acyl carrier protein (Figs 7). The synthesis of lipoic acid **7** is unusual for two primary reasons. Unlike most cofactors it is synthesized while attached to the target protein,[51,52] and the sulfur source for both thiol groups are derived from a protein associated iron sulfur cluster.[53–55]

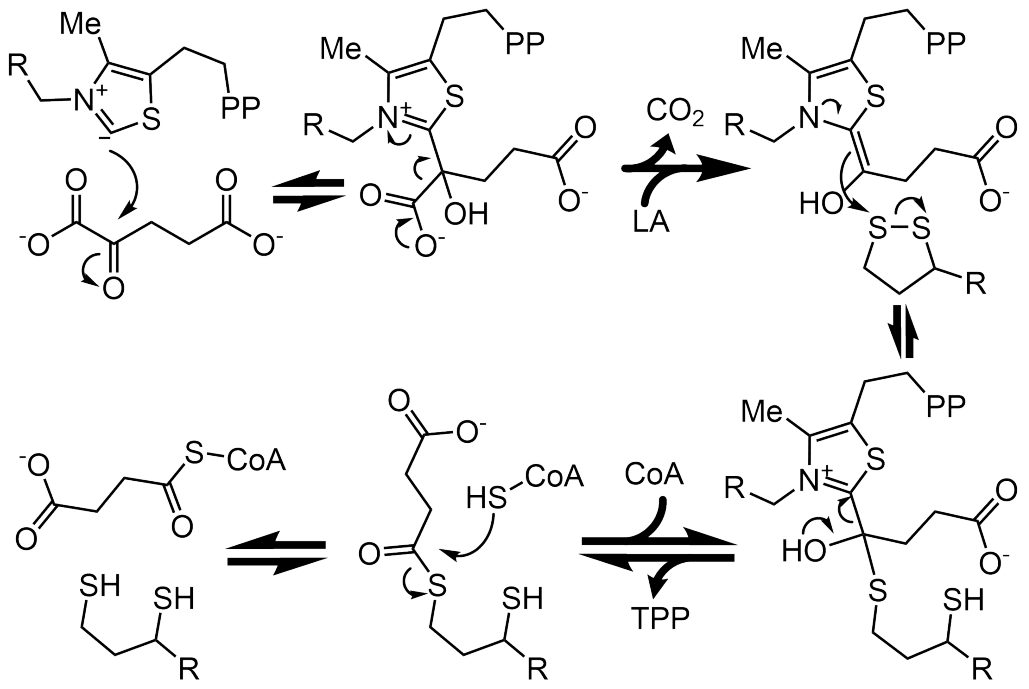

Figure 7: The role of thiamine and lipoic acid in the α-ketoglutarate dehydrogenase complex.

Octanoic acid is attached to the target lysine of E2 by LplA, which is also able to attach fully formed lipoic acid **7** to E2 that has been consumed from the environment or recycled from degraded proteins.[51] Once octanoic acid is bound to E2 **13** (Fig 8), LipA, which is also known as lipoyl synthase, is able to complete synthesis by the insertion of both sulfurs (fig 8), consuming two molecules of SAM in the process.[50,52,56] Unlike most radical SAM enzymes, LipA contains two 4Fe-4S clusters.[56,57] One of these two clusters is part of a standard radical SAM domain and is coordinated by three cysteines. It is used to reduce SAM in order to generate 5′-deoxyadenosyl radicals (Ado•) for subsequent chemical reactions. This cluster has been shown to interact with methionine in the crystal structure and a channel to the surface provides possible access for ferredoxin reductase to reduce the cluster after the reduction of SAM.[57] In contrast the second 4Fe-4S cluster is coordinated by a serine as well as the expected three cysteins. The purpose of this serine in catalysis is unknown, but it is vital to sulfur incorporation into the new

product, though not for SAM consumption.[53,57,57,58]

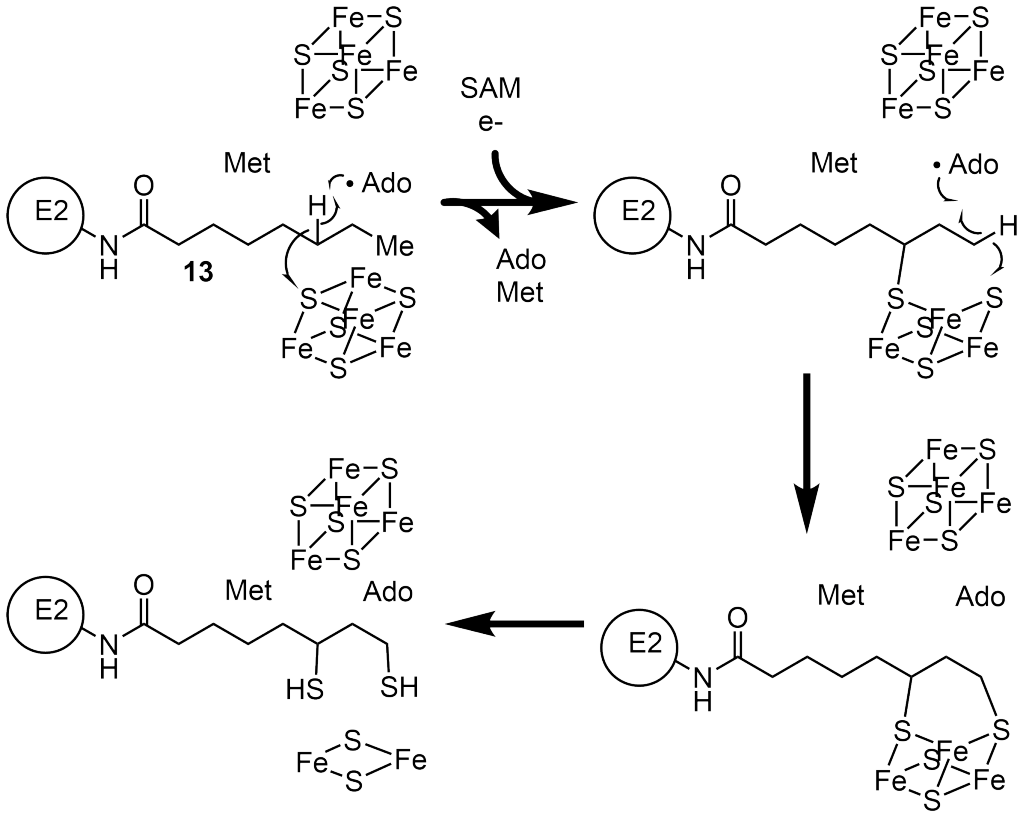

Figure 8: LipA mechanism.

A crystal structure with octanoic acid bound to the iron sulfur cluster by one covalent bond has been published (Fig 9). It occupies a small channel between the two iron sulfur clusters and, in the crystal structure, either its binding or its reaction causes the distance between the two clusters to contract from 15.3 Å to 11.8 Å, forcing the substrate and SAM into close proximity.[53]As shown in figure 8, hydrogen from carbon six is abstracted by Ado• first[52] and the resulting carbo radical binds to a sulfur on the second cluster, forming a stable C-S bond with the cluster.[50,57,59] Presumably, after the methionine and the 5′-deoxyadenosine are exchanged for a second molecule of SAM and the first cluster reduced, a second hydrogen is abstracted from the C8 position.[52] The crystal places this second carbon a little over five angstroms from either of the available sulfurs,[53]

considerably closer than the 8-10 Å predicted by earlier computer modeling.[57,58] After release of the finished product, it is unclear how the iron sulfur cluster is regenerated. It is likely that the entire protein is unfolded by chaperones to allow the iron sulfur cluster (ISC) system or another iron sulfur cluster generating systems access to LipA's active site.

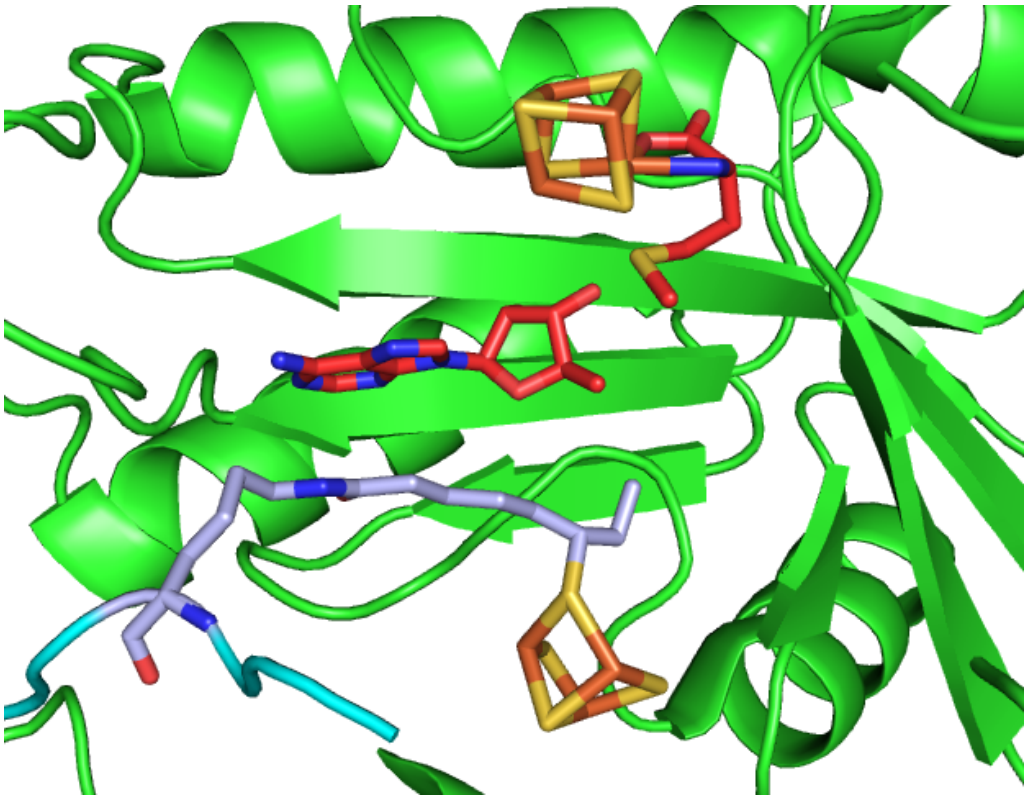

Figure 9: LipA active site with octanic acid (light blue) bound to one of the two Fe-S clusters. SAM has been cleaved to 5′-deoxy-Ado and Met (red)(PDB: 5EXK)

While LipA inserts both of the sulfurs that will form lipoic acids five member heterocycle, it does not catalyze the formation of the heterocycle. This oxidation is catalyzed by dihydrolipoamide dehydrogenase, also referred to as the E3 complex of its various systems.[60] The reduced lipoic acid probably enters a 15 Å channel formed by the dimeric protein to a disulfide motif positioned close to an tightly bound FAD.[60,61] As with other disulfie forming enzymes (Fig 2), electrons from the lipoic acid are transfered to an internal disulfide, then to FAD, and

ultimately to free floating NAD use in cellular respiration.[60,61] Mutations associated with E3 deficiency appear to predominantly affect dimerization, cofactor binding, or the conformational structure of the substrate channel.[60]

2.6 Tropodithietic Acid

Phaeobacter inhibens has a complicated relationship with the marine algae with whom it shares its environment. While the algae are actively growing this bacterium will form a commensal relationship with it, protecting the eukaryote from more aggressively pathogenic organisms. However, once the algae starts to show signs of senescence *P. inhibens* will produce algaecidal compounds such as roseobacticide to accelerate the demise of its one time partner.[62] Before this inevitable betrayal, *P. inhibens* aids its host by producing the antimicrobial compound tropodithietic acid **6**, and its tautomer, thiotropocin **15**.[63] Tropodithietic acid **6** is approximately 17-21 kJ/mol more stable than thiotropocin **15**, and the activation energy required to convert between the two forms is about 8-33 kcal/mol.[64] Tropodithietic acid **6** acts by shuttling protons and other cations across membranes, collapsing the proton gradient.[65] This compound is produced by a number of species within the *Roseobacter* genus and is significant both for its potential as a commercial antibiotic[66] and for its current benefit to aquaculture.[67,68]

The compound is synthesized from (Z)-2-9oxepin-2(3H)-ylidene-acetyl-CoA **13**, a degradation product of phenylalanine.[69] The six proteins responsible for tropodithietic acid synthesis (Fig 10) do not appear to be present in the genome of the producing organism, but rather on a low copy number plasmid maintained in the population.[68,70] The apparent sulfur source based on feeding experiments is cystine, most likely with S-thiocysteine as an intermediate.[71] The enzyme actually responsible for sulfur incorporation is hypothesized to be TdaB, which attaches the entire S-thiocysteine to compound **13**. The S-S bond is then proposed to be

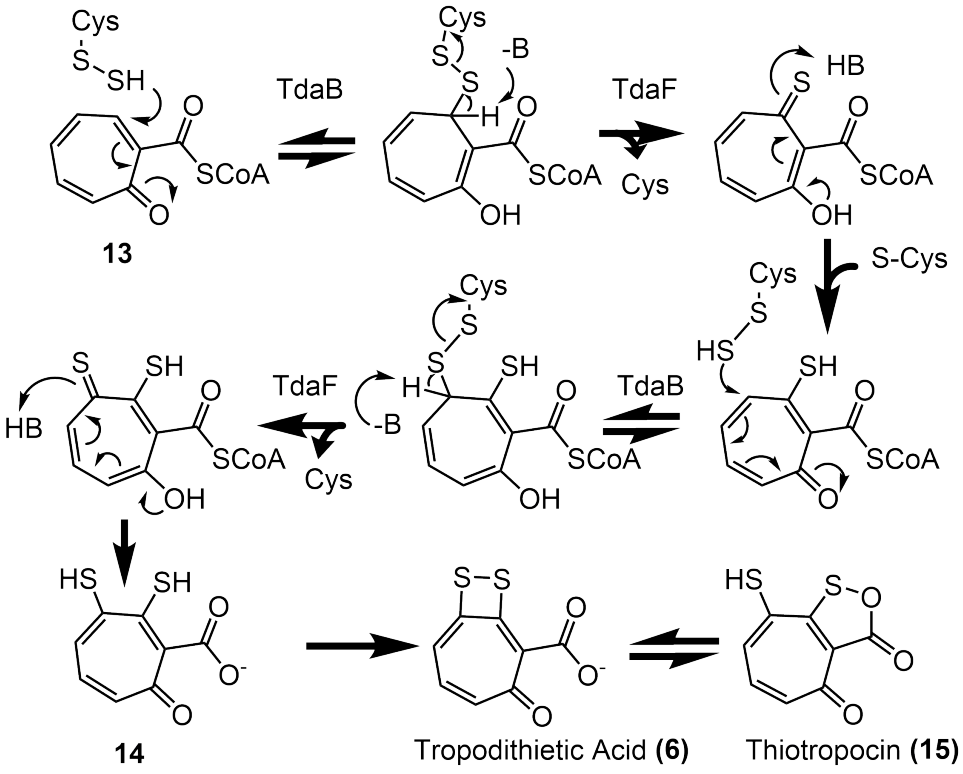

Figure 10: Proposed tropodithietic acid biosynthetic mechanism.

cleaved by TdaF. Two rounds of sulfur addition results in the formation of **14**. Neither TdaB nor TdaF have been extensively characterized and their proposed activities are based on their homology to gamma-glutathione transferases and phosphopantothenoylcysteine decarboxylases respectively.[62,71] A dedicated oxidase to close the disulfide heterocycle has not been identified. It is possible that the bacteria depends on oxygen in the environment to oxidize this bond, but it would be unusual compared to the production of more thoroughly studied disulfide containing small molecule antibiotics, which all have dedicated oxidases to close their heterocycles, as shown previously.

2.7 Asparagusic Acid

As the name would imply, asparagusic acid **3** has only been identified as a product of *Asparagus officinalis*, better known as garden asparagus. The compound

appears to be predominantly defensive, with clear anti-nematode activity and possible anti-plant and insecticidal activity.[72] Recently asparaptine, and apparent adduct of asparagusic acid to lysine, has also been identified, though its role in vivo and the enzyme responsible for attaching the amino acid remain a mystery.[73]

The carbon skeleton of this compound is derived from valine, and at least one of the sulfurs is derived from cysteine, beyond that not much is known about its biosynthesis.[72] The enzyme responsible for the closing of the heterocycle is asparagusate dehydrogenase, which use the electrons from the disulfide bond to reduce NAD. Despite its clear similarity to lipoic acid **7**, lipoyl dehydrogenase is unable to reduce asparagusic acid **3**, though asparagusate dehydrogenase can act on both compounds.[74]

2.8 Under Researched Disulfide Heterocycles

Considering the propensity of thiols to form disulfide bonds in an aerobic environment it may be presumptive to assume that a disulfide containing metabolite always has an associated oxidase to close the cycle. However, the fact that most examined compounds have an associated enzyme suggests that most understudied compounds do as well.

Nereistoxin **1** was identified as a toxic component in the marine annelid *Lumbrineris heteropoda* over half a century ago,[75] and has been shown to inhibit nicotinic acetylcholine receptors in higher organisms.[76] The relative easy of synthesis of nereistoxin **1** meant that there has not been much incentive to to study the biosynthesis of the compound,[77] particularly considering the relative difficulty in studying multicellular metabolic compounds. Its similarity to asparagusic acid **3** suggests the possibility of convergent evolution of defense mechanisms that has not been seriously explored. Gerrardine **2** was isolated from *Cassipourea guianensis* bark, and has been shown to have modest antibiotic activity.[78] Malformin **8** is produced by *Aspergillus niger* and related organisms

and has both antibiotic and antimalarial activities, though it was originally named for causing deformations in plant stems and petioles.[79] It is clearly a cyclic peptide, and based on the stereochemistry is probably produced via an NRPS, though no associated genes have been identified. It is entirely possible that the disulfide bond is produced by a promiscuous oxidase system like other disulfide containing peptides. However, having only five amino acid residues, malformin is about the same size as romidepsin **9**, opening the possibility that its disulfide bond is also oxidized by a dedicated oxidase.

3 Monosulfur Macrocycles

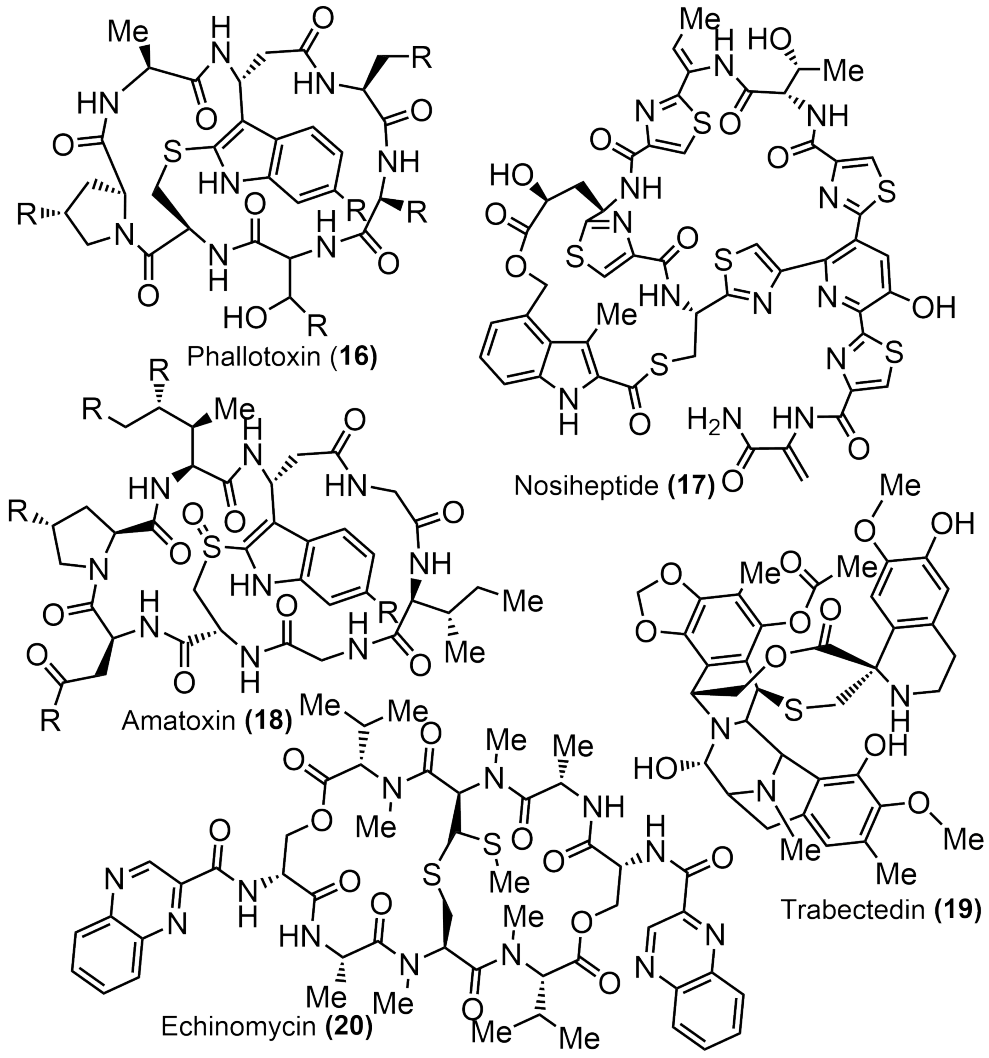

Figure 11: Macroheterocycles with a single sulfur atom.

With bond dissociation energies on the order of 300 kJ/mol, thioether bonds are somewhat more stable than disulfide bonds,[1,80] and are significantly less likely to be cleaved by reducing agents.[81] They are also more difficult for the cell to synthesize, requiring an activated carbon for the sulfur's lone pairs to attack.

While thioethers are present in a wide variety of compounds, macrocyclic compounds containing a thioether moiety are almost exclusively ribosomally synthesized and post-translationally modified peptides (RiPP). Two types of

thioethers have a well established mechanism of formation, the lanthipeptides, which link cysteine to serine or threonine through a dehydration reaction followed by electrophilic attack, and the sactipeptides, which link a cysteine to the α-carbon of another residue through a radical SAM mechanism.

3.1 Lantipeptides

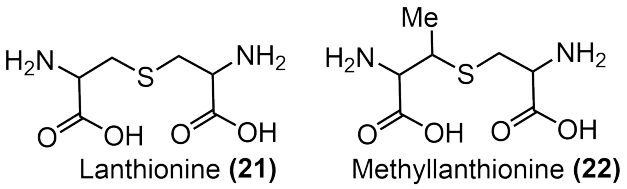

Figure 12: Lantipeptide defining moieties.

The best studied class of thioether containing RiPPs is the lantipeptides. Containing over 100 members, lantipeptides are a diverse class of cyclic peptides defined by the presence of one or more lanthionine **21** or methyllanthionine **22** moieties.[82] This class is also commonly referred to as lantibiotics, though it is unclear if all of these compounds are produced for the purpose of inhibiting competition.[83] Those that do act as antibiotics do so either by inhibiting protein production, or by binding lipid II, inhibiting its use in producing peptidoglycan, and aggregating to form membrane pores.[84,85]

As would be expected for RiPPs the sulfur source for these compounds is cysteine, which is incorporated by the ribosome during the formation of the core peptide. These residues are crosslinked to either serine or threonine by one of four different classes of lantipeptide cyclase systems. The founding cyclase for these classes are LanB and LanC for class I, LanM for class II, LanKC for class III, and LanL for class IV.[85–87] Each of these four classes appear to use a very similar mechanism for the reaction, (Fig 13), though class I synthases use two separate enzymes for the two required steps and the other three classes catalyze the cyclizations with a single multifunctional enzyme.

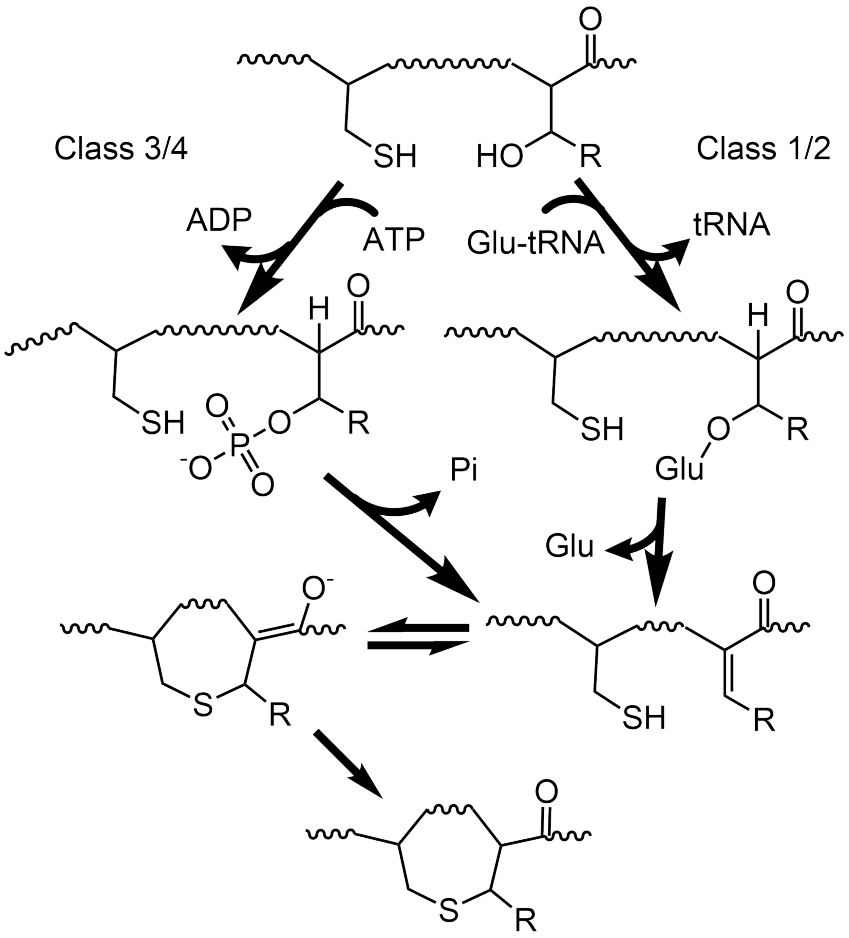

Figure 13: Overall mechanism of lantipeptide cyclases.

The first step is to activate the target hydroxyl group with a better leaving group for elimination. Class I and II use glutamate, transferred from a charged tRNA, while class III and IV use a phosphate group from ATP.[88–91] This leaving group is then eliminated by α-proton abstraction to form an electrophilic double bond. This dehydration has been demonstrated to occur even in the absence of a cysteine partner for the dehydroalanine to cyclize with.[92,93] For class I compounds this dehydrated compound is released from LanB at this point for further processing by LanC. The cyclization is catalyzed by the activation of the thiol group by a coordinated zinc, allowing it to perform a nucleophilic attack on the dehydrated residue and forming the macrocycle.[94–96] This mechanism appears to

be the case for all lantipeptide cyclases, with the exception of class III cyclases. LanKC lacks the motif for a coordinated zinc, and there is no evidence to suggest that it binds through a non-canonical coordination sphere. How LanKC activates the thiol group is still unknown.[82]

An attractive quality of the lantipeptide cyclases is the apparently generic nature of the enzyme in its activity. RiPPs contain an N terminal leader sequence that binds to the various enzymes which modify the core sequence. This ensures that only the RiPPs will be modified, and not the other proteins within the cell. Some RiPP modifying enzymes also recognize specific amino acid sequences to direct their activity to specific residues, while others will modify every residue in the core sequence that they are chemically able to. Lantipeptides are no exception, and binding of a lantipeptide cyclase to the leader sequence determines which peptides are cyclized.[92,97] It seems likely that binding this leader sequence to lantipeptide cyclases also causes conformational changes to activate the enzyme, in addition to tethering the peptide to the protein.[98,99] No peptide code directing enzyme activity to specific residues in the core sequence have been reported, though there does appear to be some optimization toward the peptide's overall structure in organisms that produce only one lantipeptide[100]. In general lantipeptide cyclases appear to be fairly promiscuous toward the core sequences they crosslink. Several organisms are capable of processing multiple peptides with little homology between them using just one cyclase system.[92,101,102] Even in organisms with only one lantipeptide, the compounds often contain multiple macrocycles with different flanking residues, all of which are processed by the same protein.[83,93]

This promiscuity also leads to a question of how the proper lantipeptide conformation is achieved, since despite having multiple possible linkages, only one pattern of thioethers is observed for each peptide. The most likely reason for this would be that the active conformation is the most thermodynamically stable. This

explanation would require the enzyme catalyzed reaction to be reversible, as has been observed for some lantipeptide cyclases.[83,103] Cyclization down the length of the lantipeptide appears to be directional, though which direction appears to depend on the system. HalM2 for example works from the N terminus to the C,[93] while ProcM works from C to N.[83] Curiously, the rate of cyclization appears to increase as HalM2 continues to act on the peptide, as would be expected if the structure was moving toward a more energetically ideal conformation, but appears to slow for ProcM. It has been proposed that the rational for this observation is that since ProcM works on multiple lantipeptides, its active site has to accommodate several different structures. As the lantipeptide's structure is restricted by the cyclizations it is less capable of interacting with the active site as well as it could when it was more flexible, slowing later reactions. By contrast HalM2 has only one substrate and can optimize its active site to accommodate the final structure.[100]

3.2 Sactipeptides

Another class of thioether containing compounds that have received growing attention recently are the sactipeptides. The family of compounds is defined by an intrapeptide crosslink between cysteine and the α-carbon of the second amino acid. At the time of this writing this small group only contains six RiPPs, subtilosin A (22) (Fig 15),[104] sporulation killing factor,[105] six cysteines in forty five (SCIFF),[106] thuricin CD,[107] thuricin H,[108] and cyclothiazomycin,[109] though genomic mining for homologous proteins suggests the there are numerous undiscovered members waiting for characterization.[107]

Biosynthesis of these RiPPs is an area of active research, but the thioether crossbridging for all of the compounds appears to involve a radical SAM mechanism (Fig 14). The 5′-dAdo radical abstracts a hydrogen from the target α-carbon,[110] the identity of this target does not appear to be catalytically

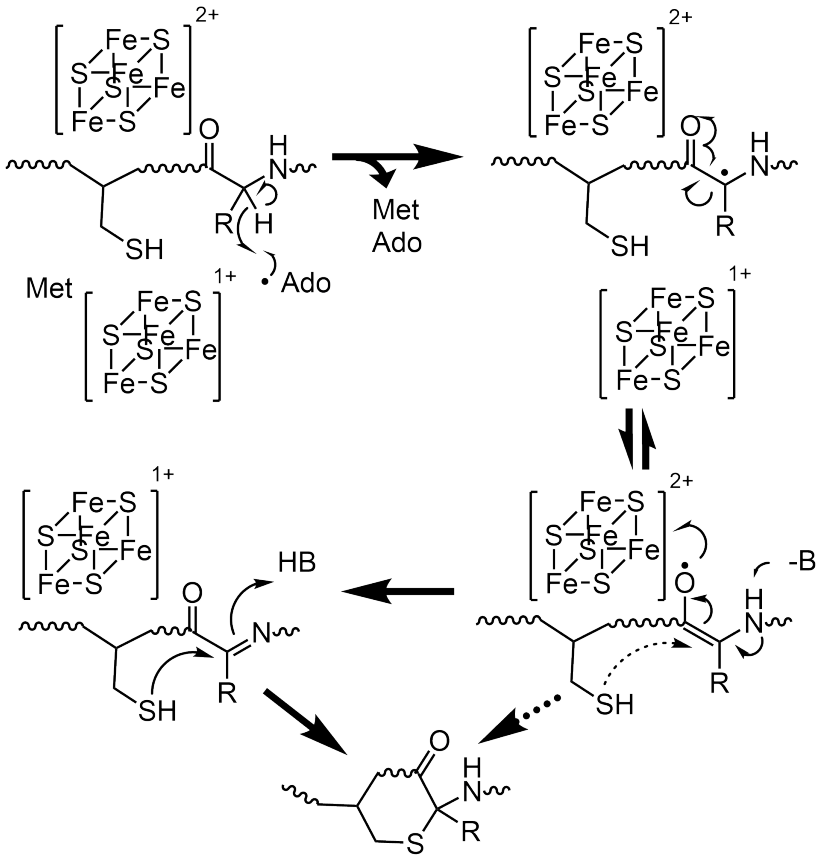

Figure 14: Overall mechanism of sactipeptide cyclases. Dashed arrows show a possible alternative mechanism.

important.[111–113] This tertiary radical probably then forms a double bond with either the keto or amino groups that then undergoes nucleophilic attack by the thiol.[114] It has also been proposed that the tertiary radical interacts with the thiol directly.[106] The thiol appears to be catalytically important to this reaction, since neither serine nor methionine can replace cysteine in the sactipeptide.[113–116]

Most of the characterized crosslinking enzymes in this family contain multiple [4Fe-4S] clusters, the exception being TrnC which contains only one [4Fe-4S] cluster and catalyzes four of the six crosslinks in thuricin CD.[117] In each of these multicomponent clusters it has been demonstrated that all of the clusters are necessary for protein activity, but only one is required for cleavage of SAM.[113,116,118] Two possible roles for the second cluster have been published. The

first is that the cluster coordinates and oxidizes the cysteine sulfur, positioning it to attack the α-carbon after 5 dAdo • activates it.[119] The second theory is that the cluster abstracts the unpaired electron from the peptide after 5 dAdo • abstracts the hydrogen, forming an electrophilic center.[114] AlbA and Tte1186a, which crosslink subtilosin A **23** and SCIFF respectively, also contain a third [4Fe-4S] cluster which is proposed to be involved in transporting the electron from the second cluster to a soluble oxidant.[106]

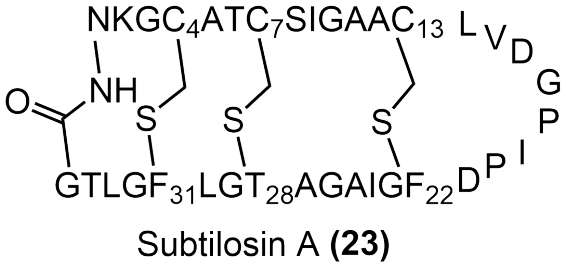

Figure 15: Cross-linkage pattern of subtilosin A **23**.

As with other RiPP treating enzymes, sactipeptide cyclases substrate specificity is based on binding the leader sequence.[112,113,116] At the intrapeptide level, linking of cysteines with target residues seems to be based on the sactipeptide's tertiary structure. For subtilosin A **23**, mutating the the non-cysteine residue involved in the thioether bond has one of two effects. Most of these mutations have no effect on the position of the thioether bonds, with the new residue forming the same bond as the wild type residue would. A few of these mutations disrupt either all three thioethers or both the C7-T28 and C13-F22 bonds, regardless of which bond was directly mutated.[111] An unnatural subtilosin A **23** analog that consists of residues 8-27 will not form the C13-F22 thioether, but an analog that consists of residues 1-6 linked to residues 27-34 through a peptide bond will form the C4-F31 thioether.[114] This suggests that the outermost linkage occurs first and that stabilizes the rest of the tertiary structure sufficiently for the next bond to be formed, though more data is necessary to determine if this outside-in order of crosslinking is a general rule, or just a quirk of subtilosin A **23**

formation.

3.3 Echinomycin

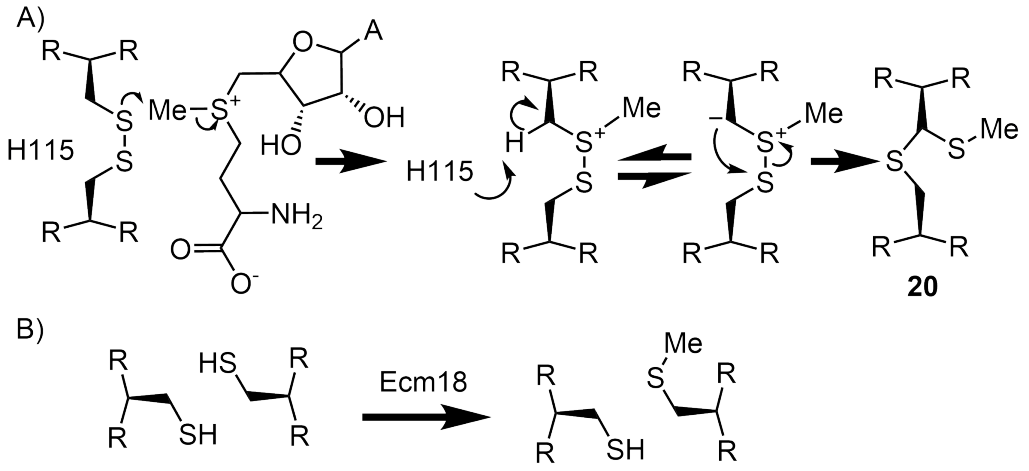

Figure 16: A) Proposed mechanism of disulfide to thioether conversion in echinomycin **20** biosynthesis. B) methylation product of the reduced substrate.

Echinomycin **20**, more archaically known as quinomycin A, is a quinoxaline antibiotic that differentiates itself from others of its class by the presence of a thioether bridge formed between two cysteine residues, bisecting the peptide macrocycle. The gene cluster for this compound has been identified in various strains,[120,121] but at the time of this writing the compound itself appears to be either unique or the founding member of a yet unidentified class of RiPPs, as no other identified RiPPs contain the same dithioacetal bridge. The bridge is first formed as a disulfide bond between the two cysteines by Ecm17, an FAD dependent oxidase similar to the previously discussed DepH (section 2.4). Afterwards, Ecm18, a radical SAM enzyme, converts the disulfide bridge to the thioether.[122] Determination of the crystal structure of Ecm18 with a non-methylated intermediate has prompted a hypothetical mechanism for this enzyme to be proposed (Fig 16 A). The conversion is initiated by the methylation of one of the two sulfurs. This allows the deprotonation of the cysteine's β-carbon

and formation of a carbanion. This carbanion undergoes a nucleophilic attack of the second cysteine, resulting in a rearrangement that forms the thioether and a methyl sulfide. This mechanism is supported by the isolation of a monomethylated product when a monocyclized (reduced dithiol) substrate is fed to Ecm18 in place of the reduced bicycle (Fig 16 B).[81]

3.4 Indole Linked Macrocycles

Nosiheptide **17** is an RiPP antibiotic produced by *Streptomyces actuosus*.[123] In addition to the numerous thiazole rings, the formation of which will be discussed in section 4, the compound contains an unusual indolic acid derived macrocycle (Fig 17). The sulfur source for this heterocycle seems to be from cysteine incorporated into the initial peptide. The tryptophan however is not present in the core sequence and seems to be added separately to form the cycle. Research is ongoing, but initial results suggest that an indolic acid **24** is loaded onto NosN by the activity of NosI. NosK then transfered it to the cysteine side chain of the thiopeptide. Once indolic acid is connected to the rest of the peptide, NosN will catalyze both the methylation of the aryl ring and the closure of the macrocycle within the substrate.[110] This final enzyme is of particular interest as a radical SAM enzyme which binds two equivalents of SAM at once, one is probably the methyl donor while the other abstracts a hydrogen.[110] While Figure 17 shows a monomethylated indolic acid **24** being attached, it is unclear whether this methylation occurs before, during, or after macrocyclization.[124]

While the enzymes involved are not as well researched, the amatoxin **18** and phallotoxin **16** classes of compounds deserve some note here. Amatoxins **18** and phallotoxins **16** are the compounds responsible for toxicity in the aptly named death cap mushroom, or *Amanita phalloides*.[125] Both of these toxins are RIPP compounds[126] with the primary differences being that amatoxins **18** are octapeptides containing a sulfoxide thioether bridge, while phallotoxins **17** are

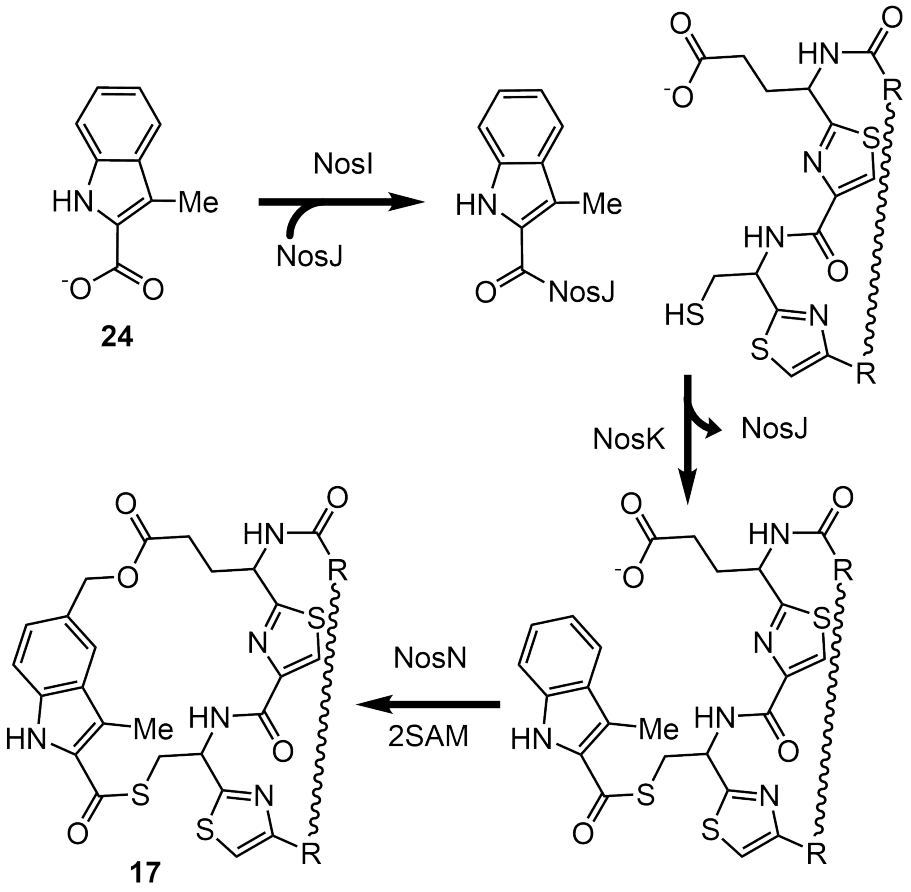

Figure 17: Proposed mechanism of nosiheptide bicyclization. The process is shown starting with a methylated indole **24** though it is unknown whether this methylation occurs before or after the macrocycle is formed.

heptapeptides that use a sulfide thioether instead, both of which contain two macrocycles.[127] The larger cycle formed by a peptide bond between the first and last amino acids in the peptide, catalyzed by a prolyl oligopeptidase type serine protease.[128] A smaller cycle is formed by a thioether bridge between the R groups of a cysteine and tryptophan. The dissimilarities between the residues used to form the thioether makes it unlikely to use the same mechanism as either lantipeptides (Fig 13) or sactipeptides (Fig 14), and the fact that the indole is clearly part of the core peptide makes a nosiheptide (Fig 17) like mechanism equally unlikely. Unfortunately, the inability to culture producing fungi in the lab has inhibited research into these compound's biosynthesis, and the gene

responsible for formation of these unique macrocycles remains unknown.

3.5 Trabectedin

Trabectedin **19**, also refereed to as ecteinascidin 743, is a clinically useful anti-cancer compound whose mode of action is inducing formation of double stranded DNA breaks after binding to the minor grove.[129] As a natural product trabectedin **19** is exemplary of many of the challenges facing the field of metabolomics. It is a relatively complex compound, consisting of three tetrahydroisoquinoline moieties each with different individual modifications, and it is produced by an organism associated with *Ecteinascidia turbinate* that cannot be independently cultured in the lab. Metagenomics data has allowed researchers to identify a likely gene cluster, despite being unable to obtain a pure culture of the producing organism. It was also possible to confirm that the cluster's genes are translated into their protein products. Using the codon preferences of this gene cluster they were able to identify *Candidatus Endoecteinascidia frumentensis* as the most likely producing organism.[130]

One primary obstacle to this metagenomics approach is the difficulty in expressing proteins heterologously to confirm their action. Most relevant to this review is the lack of information concerning the thioether cycle in the product. The sulfur source has been confirmed as cysteine by radiolabeled feeding experiments,[131] and this is consistent for the predicted amino acid substrates of the NRPS protein EtuA3.[130] The protein responsible for forming the thioether or its mechanism of action however has yet to be determined.

4 Thiazoles, Thiazolines, and Thiazolidines

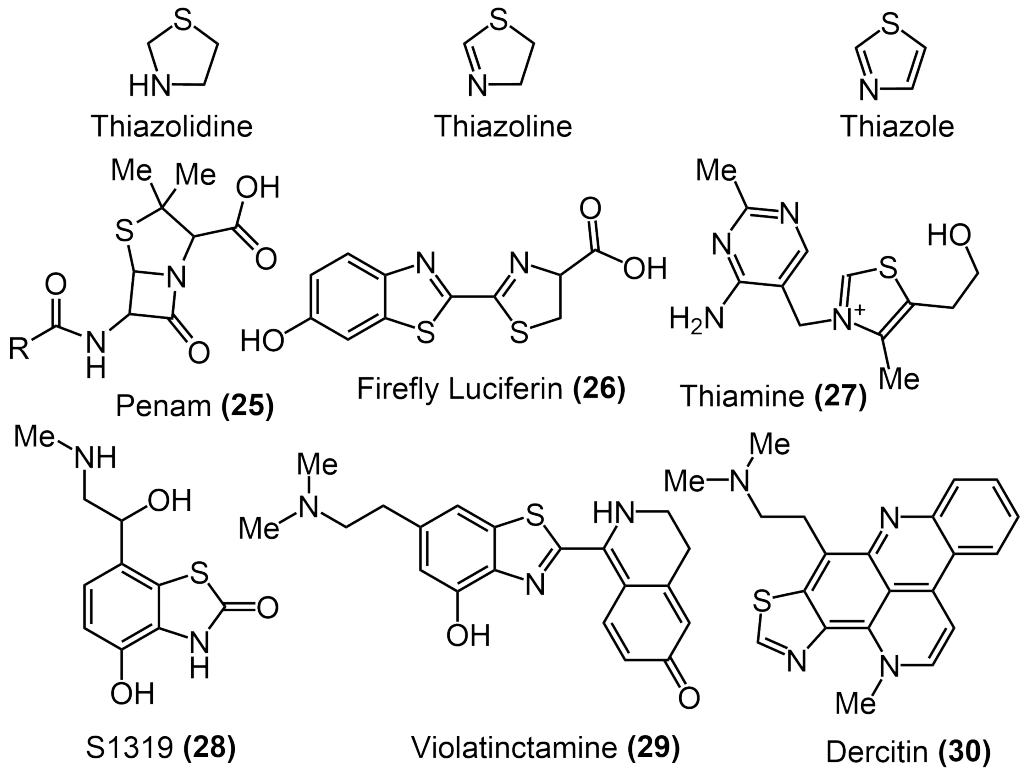

Figure 18: Metabolites containing thiazole, thiazoline, or thiazolidine rings.

Thiazole rings are five member heterocycles including a sulfur, nitrogen, and two double bonds. Reduction of one or both double bonds results in the formation of thiazoline and thiazolidine respectively (fig 18). Thiazole and thiazoline are able to participate in a diverse array of reaction, with two nucleophilic centers at the nitrogen and sulfur and the carbon of the C=N bond able to act as an electrophile.[132,133] The greater aromaticity of thiazoles compared to other heterocycles such as pyrrole or oxazole,[134] allowing for cells to produce relatively stable ring structures while still having lone pair electrons from the sulfur. As such it should be unsurprising that biology makes use of these rings both as structural elements and reactive centers.

Five different methods of forming these moieties can be said to be well

understood, namely their incorporation into NRP, RiPP, Penam **24** products as well as the formation of thiamine **26** in both eukaryotes and prokaryotes. For NRPs, RiPPs, and penams **24** a cysteine thiol is activated by base or iron and attacks a keto group of an adjacent peptide bond (Figs 19, 20, 23). NRP and RiPP systems are particularly promiscuous in the peptide sequences they are able to cyclize, while penams can only be formed from a tripeptide substrate, L-alpha-aminoadipic acid-cystine-valine (ACV). Thiamine sulfurs are also derived from cysteine, directly from the catalyzing enzyme in eukaryotes (Fig 28), and indirectly from a thioacid carrier protein in prokaryotes (Fig 26).

4.1 Peptide Thiazoles, Thiazolines, and Thiazolidines

The variety of available amino acid side chains and the highly mobile amide backbone of polypeptides allows for innumerous three dimensional structures to be constructed from this polymer. In the face of such diversity it is unsurprising that useful molecules, such as antibiotics and chelators, can be constructed from small peptides. Unlike macromolecular proteins however, these peptides are usually modified further, in order to restrict which conformations are favored to those which are most useful for the producing organism. It would be impossible to ensure the proper conformations of these small peptides using canonical residues, since there are so few residues available to interact with each other.[135,136] The formation of a thiazole ring, or its oxidized varients, is common in peptides. These pentameric rings help to form the proper coordination geometry around the target ions in chelators,[136–139] and can make antibiotics resistant to hydrolysis, improving the effectiveness of antibiotics.[140] Thiazolines and thiazolidine are particularly useful in chelators due to their greater flexibility, allowing them to make fine adjustments to better coordinate the target metals[136]

Two primary pathways exist for the formation of peptide metabolites (Figs 19, 20). As discussed in section **3.2** RiPPs are formed in the ribosome using

mRNA as a template, and then further modified by other enzymes. This mode of biosynthesis usually has the advantage of greater variability and faster adaptability, since modifications only require mutations in the gene encoding the core peptide.[137] In contrast, NRPs are formed through the action of dedicated modular protein complexes reminiscent of PKSs, with each protein unit catalyzing the addition of one specific amino acid to the growing chain. Because changes involve the exchange of large subunits, it is more difficult to alter the sequence of NRPs compared to RiPPs. However, NRPSs have the advantage of being able to incorporate amino acids beyond the standard twenty into the growing polymer.[141] Both of these systems use similar underlying chemistry to incorporate thiazoles into the final compounds, but there are several key differences in the overall processes.

The standard NRPS unit includes three subunits. An adenylation domain which selects and activates the next amino acid, then transfers it to the phosphopantetheine arm of the peptide carrier protein (PCP) domain. The growing strand is transferred to the PCP domain when the condensation domain forms the new amide bond between the growing strand and this new residue. The PCP domain holds the peptide for more variable associated subunits to act on it, before passing the peptide to the next step in the complex.[142] The heterocyclase domain, which forms the thiazoline moiety, has high sequence homology to condensation domains, and can often replace them in the complex, acting as dual activity condensase/heterocyclase enzymes. Despite its ability to replace condensation domains, heterocyclase domains lack the HHXXXDG sequence conserved in condensation domains. Instead they have a conserved DXXXXDXXS sequence.[143–145] After the thiazoline is formed, it can then be reduced or oxidized to thiazolidines or thiazoles respectively by a separate redox protein.[146–148]

The mechanism for the formation of thiazoles (Fig 19) appears to be identical to the formation of oxazoles in NRPs. It has been demonstrated that for

certain systems, replacing the cysteine adenylase domain for a serine results in the heterocyclase domain creating an oxazole.[139,147] Although there has been some indirect evidence suggesting that the initial bond formed by the heterocyclase domain is a thioester,[149] it is widely accepted that the first step in creation of an NRP thiazole is the formation of a peptide bond. This is supported by the fact that N900A and S984A mutations in MtaD, a duel condensation/heterocyclase domain, was able to lengthen the polypeptide chain without the cyclization, resulting in the presence of free thiols in the product.[146] After the peptide bond is formed a basic residue is proposed to deprotonate the thiol group of the cysteine, making it a better nucleophile for attack on the α-carbon of the ketol group to form the five membered ring. A second base removes a hydrogen from the nitrogen, allowing the formation of a double bond and excluding a water molecule.[143,146] At first glance it seems likely that these basic residues involved in catalysis could be part of the conserved DXXXXDXXS. This speculation has not been supported by experimental evidence. Interestingly, the heterocyclase domains of AngN (anguibactin),[150] MtaD (myxothiazol),[146] and EpoB (epothilone)[140] could each loose either of their conserved aspartates and still produce the thiazole containing product without any reported uncyclized peptides, though overall production was significantly reduced. This suggests that the DXXXXDXXS motif is either not the catalytic domain, or that there exists a redundant catalytic residues in the subunit.

The thiazole/oxazole-modified microcins (TOMM) class of compounds are a set of RiPPs that have oxazole and/or thiazole moieties added to the peptide post-translationally.[151] Like NRPS heterocycles, there seems to be a degree of overlap in the activities of thiazole and oxazoles forming proteins, and some heterocyclases are able to form both, though in these cases the thiazole rings are formed more efficiently.[137,152] Like all RiPPs the initial polymers are produced by the ribosome in an unmodified form, meaning that unlike NRPs, the thiazole has

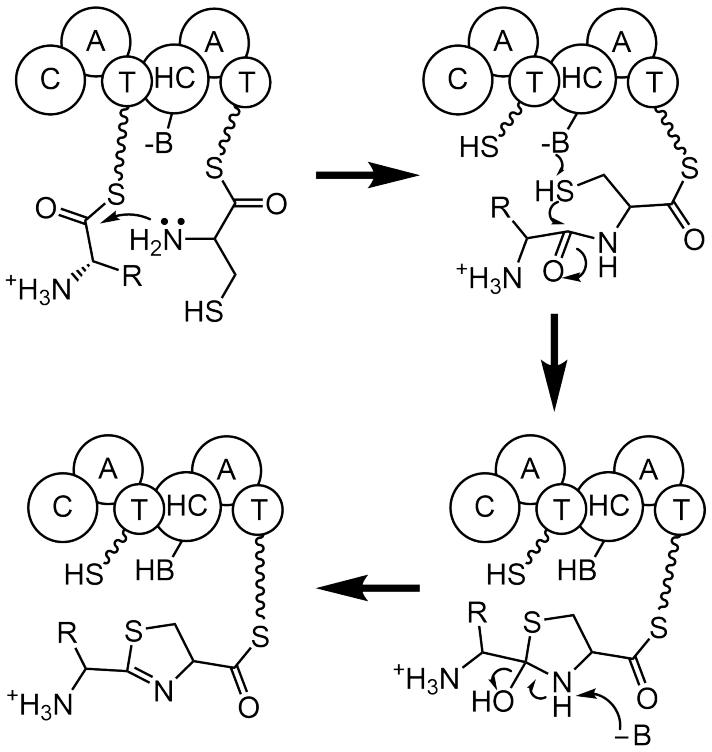

Figure 19: Mechanism of NRPS heterocyclase domains.

to be added to a free-floating polypeptide. While a great deal of research has been done on various TOMM compounds, the heterocyclase that has the most data relevant to this review is the BalhC/D complex, involved in the synthesis of TOMMs in *Bacillus*.

The heterocycle is formed predominantly by BalhD (Fig 20), with BalhC improving its activity, possibly by better orienting the substrate in the active site.[153] As in NRPSs, the ring is most likely formed by a nucleophilic attack from the thiol group against the ketol carbon of the N terminal amide bond. Unlike NRPS heterocyclases, BalhC/D and related enzymes consume ATP in the process. It is proposed that thiazoline formation is first catalyzed by a basic residue activating the thiol group. This reversible reaction is driven forward by a subsequent adenylation of the resultant oxygen anion by ATP. The reaction is completed by the abstraction of a proton from the nitrogen, forcing the release of

AMP as the thiazoline is formed.[153,154]

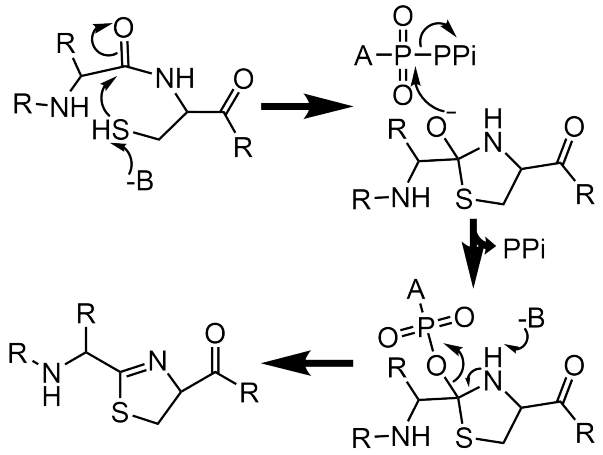

Figure 20: TOMM thiazole formation.

In addition to the catalytic mechanism, how RiPP modifying enzymes select for the proper residues to modify is an important question. It is fairly clear that, like other RiPP enzymes, the primary source of specificity for TOMM modifying enzymes is the leader peptide, which often acts to anchor peptides to requisite enzymes during processing,[137] however the extent of the leader peptide's importance does not appear to be the same for all RiPPs. TruD for example has been reported to be capable of specifically cyclizing one of its conjugate trunkamides heterocycles without a leader peptide. This opens the possibility that, in trunkamide's case at least, the role of binding to the leader peptide ensures processivity, but is otherwise not important to enzyme activity.[155]

There also appears to be specificity signals determining which residues within the core peptide are modified. Microcin B17 processing, for example, requires a glycine immediately before the cysteine or serine being cyclized.[137] Thiocillin, patellamide, and various other TOMMs lack this signal, so it is clearly not universal in the biosynthesis of TOMMs (Fig 21). In thiocillin, modification of residues flanking cystine in thiocillin did not prevent natural cyclization. However, cysteines added to the thiocillin core peptide failed to form a thiazole, suggesting a specific signal in the core peptide that is not immediately N or C to the cyclized

residue.[156] These heterocyclization signals also appear to be independent of other post translational processing, such as macrocyclization.[155]

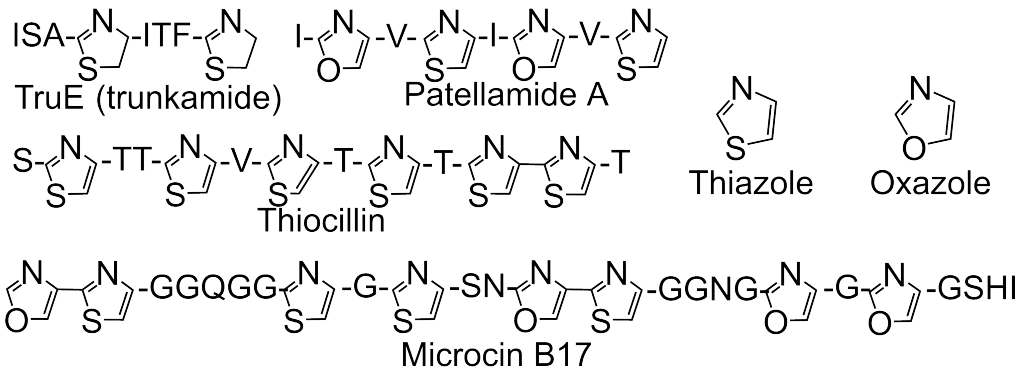

Figure 21: Modified core peptides of TOMMs cited in this section and the structure of the oxazole moiety for comparison, additional TOMM modifications not shown.

4.2 Penams

Discovered in 1928 by Dr Fleming, penicillin has not only saved millions of lives directly as the first isolated antibiotic opened up fields of research which have given us the variety of antimicrobial compounds which we enjoy today. Even with such a long and prodigious history of research, study on penicillin biosynthesis continues to yield interesting results to this day. Excluding the monobactam class, all β-lactam antibiotics also contain either five or six-member ring fused to the β-lactam.[157,158] penam **25** and cepham antibiotics contain a thiol group in their bicyclic core, and are therefore the two classes that will be discussed in this review.

β-Lactam antibiotics are named after the four membered amide heterocycle necessary for their activity. This β-lactam moiety gives the compound very similar conformation to the D-Ala-D-Ala found at the terminus of peptidoglycan's pentapeptide. This allows β-lactams to bind to enzymes that interact with this dipeptide moiety,[159] most notably the transpeptidases. Transpeptidase crosslinks the strands of peptidoglycan in bacterial cell walls by first forming a covalently linked intermediate with the tetrapeptide (the terminal D-alanine is displaced by

the enzyme) and then forming a peptide bond between the remaining D-alanine and an amino group on the lysine or diaminopimelic acid (DAP) functional group. β-Lactams covalently link to the transpeptidase by the same mechanism as the pentapeptide, but where the pentapeptides terminal alanine is displaced by the enzyme, only the amide bond of the β-lactam bonds is disrupted. The resulting acyclic adduct remains bound to the active site and the bulky side chains prevents interaction with another pentapeptide, which would otherwise dislodge this covalent adduct and release transpeptidase (Fig 22).[160]

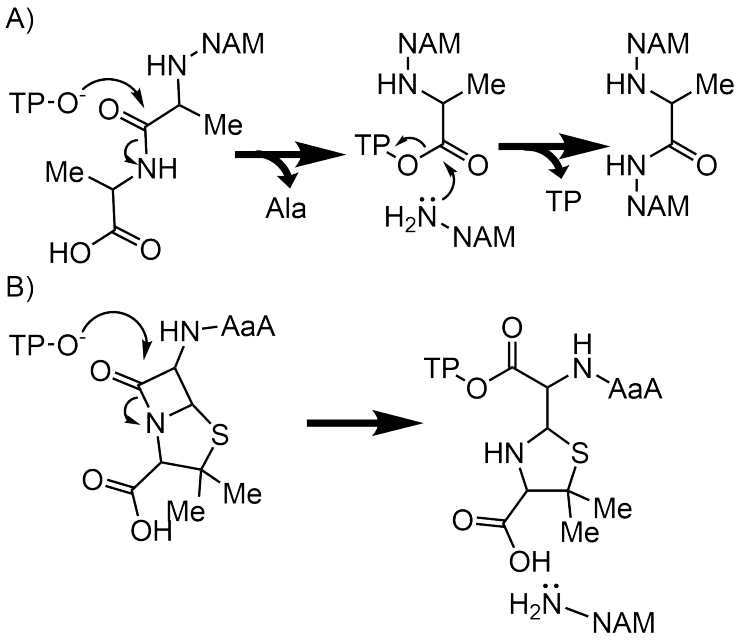

Figure 22: A) Normal transpeptidase activity B) Mechanism of β-lactam transpeptidase inhibition.

Both penams **25** and cephams are synthesized through a common intermediate, isopenicillin N (IPN) **31**, by the enzyme IPN synthase (IPNS),[161,162] which uses the cysteine derived thiol to form a ring in the mechanism (Fig 23). Monobactams and carbapenems in contrast use a metal independent ATP consuming mechanism to form their β-lactam moiety.[157] IPNS is a non-heme mononuclear enzyme that catalyzes the bicyclic ring formation through an oxygen-dependent oxidation of an aminoadipoyl-cystein-valine (ACV) tripeptide.

Four electrons are oxidized during the conversion of ACV to **31**, producing two molar equivalents of water and consuming one molar equivalent of dioxygen.[163]

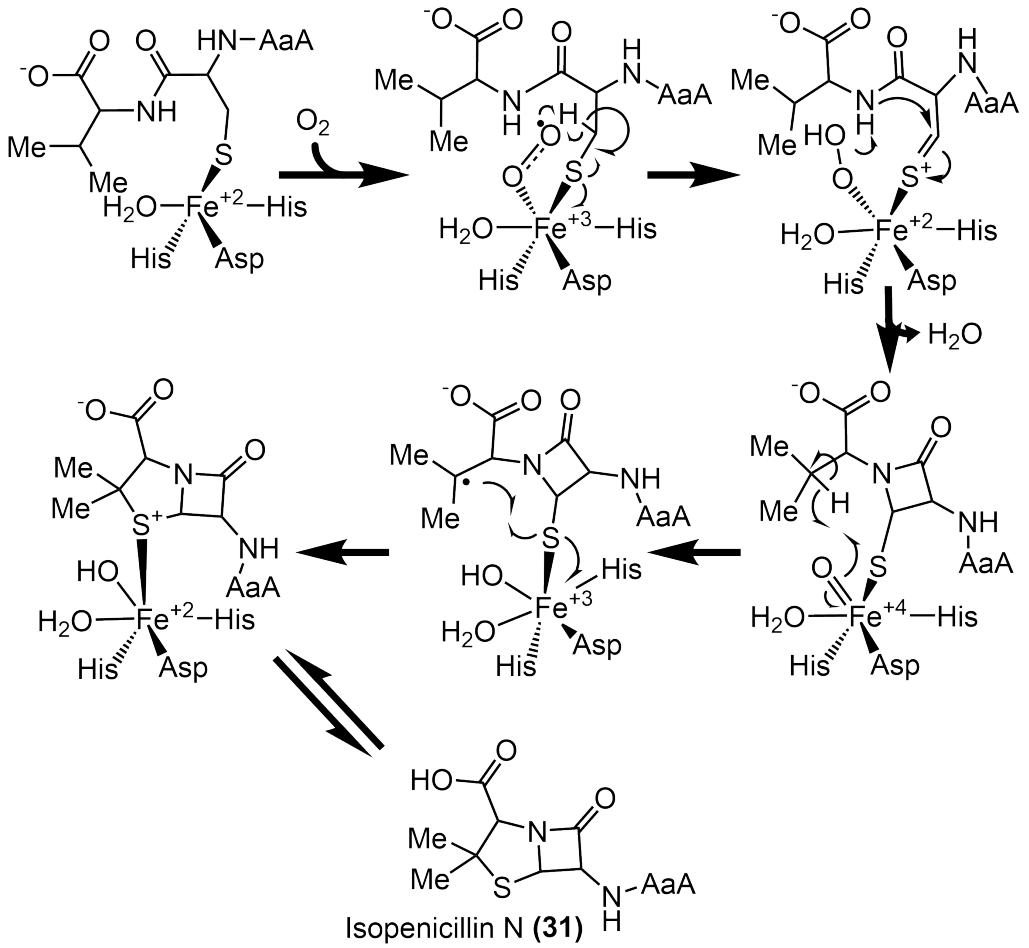

Figure 23: Mechanism of IPNS.

Structurally as well as functionally IPNS is highly similar to the alpha keto gluterate (AKG) dependent family of non-heme oxidases, with a core jelly roll beta barrel motif coordinating the iron center.[163] Specifically, His270, His214, and Asp216 make up the 2His/1Carboxy motif used to coordinate the iron.[163] Prior to substrate binding this iron center is in a high spin state with octahedral geometry and Gln330 occupies the space where the cysteine of the ACV substrate will bind, coordinated through its side chain oxygen.[164,165] Binding of ACV occurs before oxygen coordination, with the cysteine displacing Gln330 at the position trans to

His270.[158,166,167] The aminoadipoyl and valine occupy hydrophobic pockets that are fairly accepting of other hydrophobic moieties.[168] It has been proposed that Arg279 and Ser281 are important in coordinating the terminal carboxyl group of the ACV substrate, preventing it from interacting with the iron center where it would otherwise block oxygen binding.

The binding of the ACV substrate to the iron center through the thiol group of ACV induces oxygen binding trans to Asp216 through two apparent mechanisms. First, the thiol reduces the iron center from Fe(III) to Fe(II), making it energetically more favorable for oxygen to bind.[169] Second, the hydrophobic valine side chain helps to displaces a water molecule from the apparent oxygen coordination site around the iron by forming a hydrophobic pocket for the oxygen.[170] The binding orientation of ACV before and after oxygen binding is well supported by crystallography using NO as a non-reactive homolog for O_2.[163]

The peroxo formed from the oxygen binding to iron is proposed to abstract a hydrogen from the β-carbon of the cysteine amino acid.[171] This is one of two partially rate limiting hydrogen abstractions as determined by deuterium kinetic isotope effect (KIE) studies.[168,172] The radical formed from this abstraction quickly forms a nucleophilic double bond with the near by sulfur, temporarily breaking its covalent bond with the iron center. The predominant theory is that the hydroperoxo abstracts the proton from the nitrogen group on the valine, causing it to undergo an electrophilic attack on the cysteine β-carbon, closing the β-lactam and reestablishing the covalent bond with the sulfur. There is some confusion over the closing of the β-lactam, as it has been shown that it is more favorable for the hydroperoxo intermediate to abstract a proton from a nearby water rather than the ACV nitrogen,[169,171] but no good alternative base has been proposed in its place to drive the β-lactam ring closure. The formation of the β-lactam ring and subsequent formation of the iron IV oxo IPNS intermediate is supported by computational modeling[169] as well as the direct observation of a β-lactam

intermediate in crystals.[173]

Regardless of what protonates the hydroperoxo, the result is an Fe(IV) oxo species.[174] This species is necessary for the otherwise unfavorable abstraction of a hydrogen from the β-carbon of the valine, the second partially rate limiting abstraction identified from KIE studies.[168,172] This forms a tertiary radical on the valine. This radical attacks the cysteine sulfur, closing the five member ring and displacing the bond with the iron, allowing for the IPN **31** to dissociate from the enzyme.[162,168]

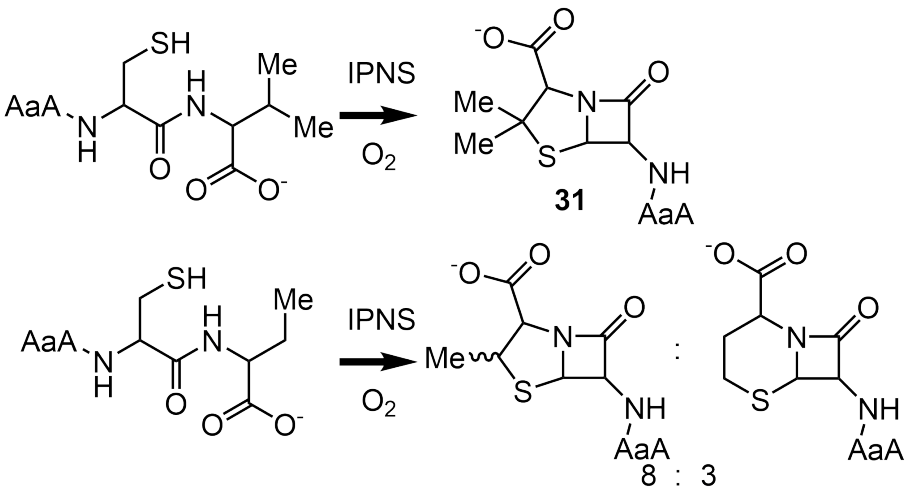

Figure 24: Products of D-α-aminobutyrate containing substrate.

The generation of a radical in this final step has been confirmed through various reactive analogs of valine. IPNS has shown an ability to accept a variety of hydrophobic valine analogs in the ACV substrate, as well as a surprising lack of specificity in where it generates the carbon radical.[162,168,170,175] Replacement of valine with D-α-aminobutyrate generates both R and S stereochemistries for the resulting penam **31** in an approximate ratio of 1:7 (R:S) (Fig 24).[170] This experimentally supports the loose binding of the valine observed in the crystal structure, where the abstracted hydrogen is oriented away from the iron ion center upon the initial binding of ACV and then must rotate around to point the hydrogen toward the center once NO has bound. More surprisingly, this analog

also generates a cepham product in a ratio of 3:8 cepham to lactam.[170] This suggests that the iron (IV) oxo species is capable of generating a radical on any of the three carbons in the valine but only abstracts from the β-carbon primarily because of the relative stability of the tertiary radical. Thus, the β-radical it is the only catalytically relevant radical, as opposed to selectivity being determined by substrate orientation by the enzyme.

4.3 Thiamine

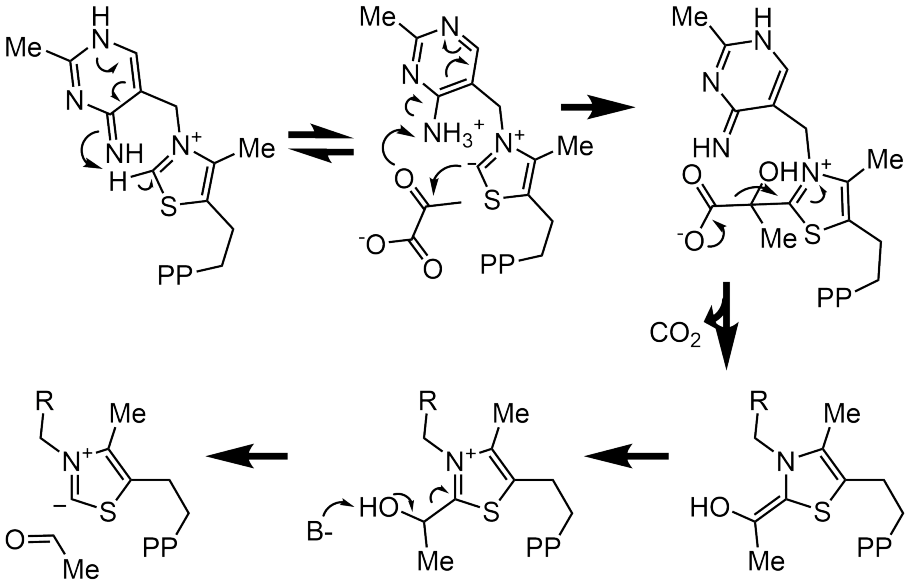

Figure 25: Thiamine's mechanism in pyruvate dehydrogenase.

Thiamine **27** is a cofactor involved in the transfer and removal of carboxyl groups across all domains of life, and is vital for the metabolism of amino acids and carbohydrates in the cell.[176] The thiazole **27** acts as the point of attachment for substrates, with the aminopyrimidine likely acting as a base to directly or indirectly activate the thiazole **27** by forming the nucleophilic ylide (Fig 25).[177]

Thiamine **27** is produced by numerous species of bacteria, fungi, plants, and archaea, however mammals have lost the ability to produce the compound and must obtain it through their diet.[178,179] While thiamine **27** has the distinction of

being the first identified vitamin, discovered by Dr. Suzuk in 1935,[180] it hasn't been until the early part of the twenty first century that the biosynthetic pathway has been pieced together. The thiazole and pyrimidine heterocycles are synthesized separately before being linked together to form thiamine. As this review is focused on the formation of thioheterocycles, only the formation of the thiazole phosphate carboxylate **34** component will be discussed.

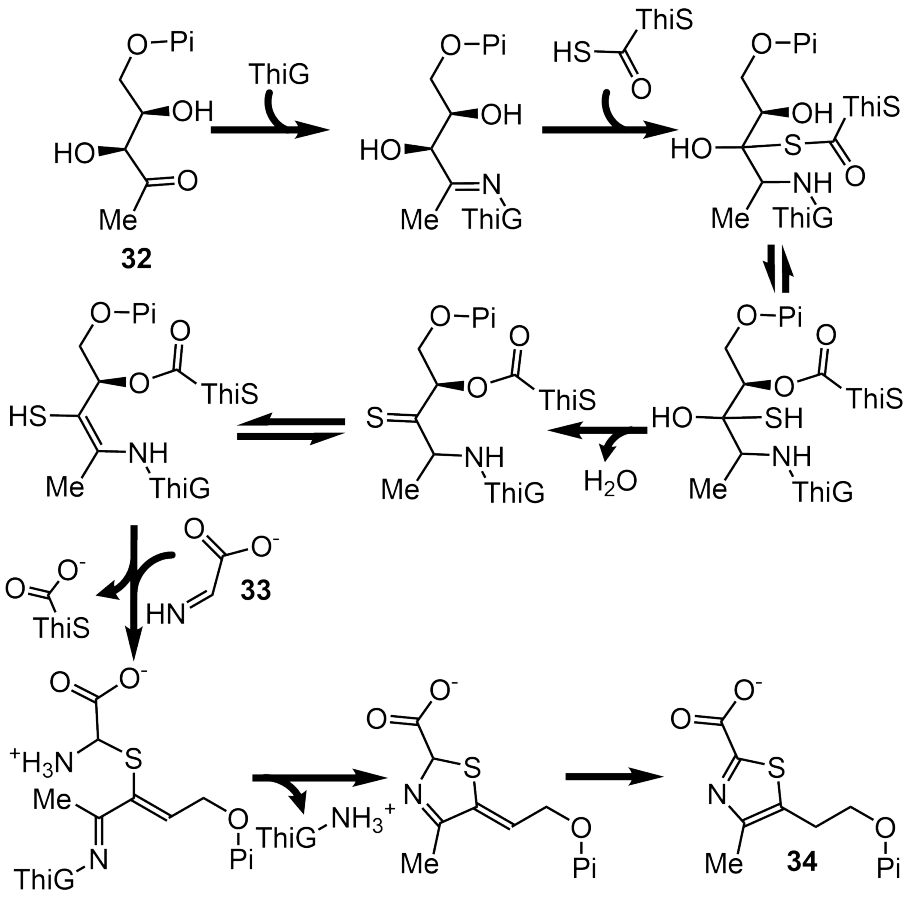

Figure 26: The mechanism of thiazole phosphate carboxylase **34** biosynthesis in prokaryotes.

In all bacteria where thiamine synthesis has been studied, predominantly *Escherichia* and *Bacillus* species, the formation of thiazole phosphate carboxylate **34** involves the activity of six proteins acting to combine sulfur, deoxy-D-xylulose phosphate **32**, and dehydroglycine **33** (Fig 26).[181] The first step for sulfur

incorporation is the loading of a thiol group onto the C terminus of ThiS. This is catalyzed by ThiF, which forms a surprisingly stable complex with ThiS to adenylate its C terminus prior to thiol donation by a cysteine desulfurase.[181–187] The most commonly used cysteine desulfurase identified in this reaction is the promiscuous IscS, though it has also been shown that many organisms have redundant cysteine desulfurases, such CdsH in *Salmonella enterica*, that can be used to load ThiS.[188,189] ThiG is the primary enzyme catalyzing the formation of a thiazoline phosphate carboxylase **34** product from deoxy-D-xylulose-5-phosphate **32**, dehydroglycine **33**, and the thiolated ThiS.[178,181,183,184,190] The deoxy-D-xylulose-5-phosphate **32** used by ThiG is synthesized from pyruvate and glyceraldehyde-3-phosphate by Dxs, while the dehydroglycin **33** can come from various sources, depending on the organism and environmental conditions. In *Bacillus subtilis* a glycine is dehydrated by ThiO to form dehydroglycin **33**, while in *E. coli* under anaerobic conditions a tyrosine is degraded by the radical SAM ThiH to generate the two carbon compound.[181,190–192] A reasonable intramolecular mechanism for ThiG has been proposed and is supported by numerous experiments and the crystal structure,[180,193] though the final cyclization step still requires confirmation. The sugar is bound to the enzyme by Lys96 at C2.[183] This induces an Amadori-type rearrangement, converting the alcohol at C3 to a carbonyl.[194] This allows a nucleophilic attack by ThiS to deposit its thiol at the C3 position, displacing the oxygen. The dethiolated ThiS then undergoes a S→O acyl migration to C4 before being releases to be reloaded with another thiol.[183,184] This thiol can then attack the dehydroglycine at C2. The last step is the displacement of lysine 96 by the dehydroglycine amino group, closing the heterocycle and releasing the enzyme. The thiazoline ring can then tautomerize nonenzymatically into the requisite thiazole, though TenI usually accelerates the tautomerization in vivo.[179,195]

In contrast to the prokaryotic system, to make thiazole phosphate

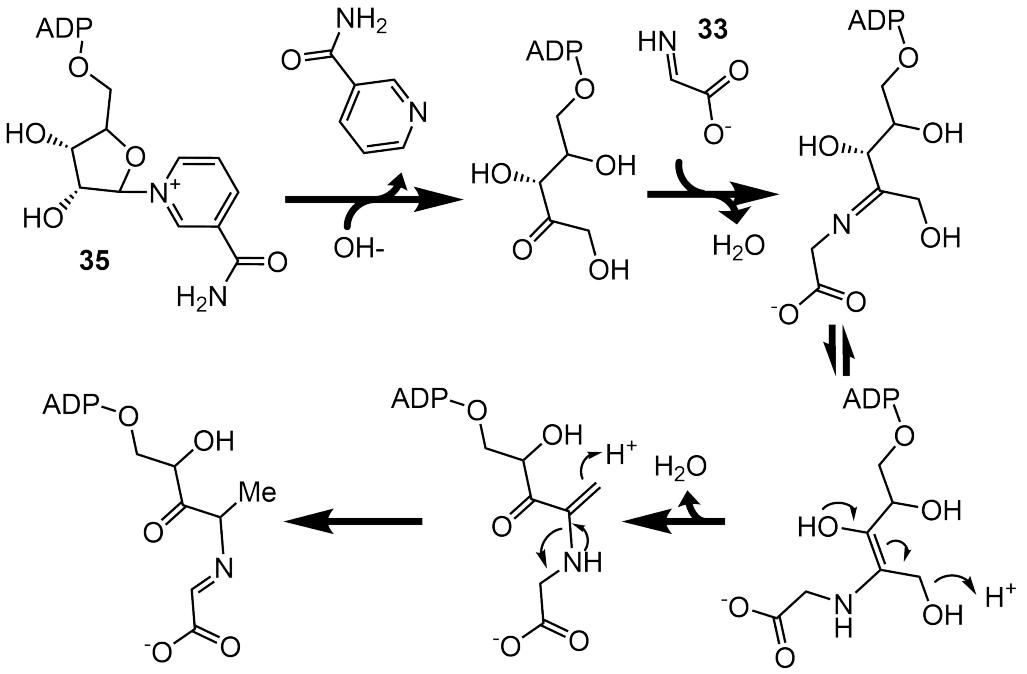

Figure 27: The iron independent Thi4 reactions.

carboxylate the eukaryotic/archaeal system requires just one protein, Thi4, and two substrates, dehydroglycine **33** and NAD^+ **35** to form thiazole phosphate **34** carboxylate (Figs 27, 28).[176] Thi4 is an iron-dependent enzyme which forms octameric complexes in solution.[196] The protein also has what appears to be a degenerate FMN binding site, though no one has found FMN in the protein and the structure suggests that it would be impossible for the cofactor to bind.[197]

The Thi4 reaction begins with the hydrolysis of NAD **35** to remove the nicotinamide moiety.[198] A dehydroglycine **33** is then added to the freed sugar moiety through a dehydration reaction.[198] Another dehydration reaction removes a hydroxal group concurrent with sulfur attachment. The exact mechanism of heterocycle closure has yet to be confirmed, though reasonable mechanisms have been proposed (Fig 28).[179,196] Hydrolysis of the diphosphate linker with the adenosine leaves thiazole phosphate carboxylate for further reactions.[179]

With so many different reactions catalyzed by one enzyme it is no surprise that Thi4 remains an enzyme of interest. Primary work in recent years has focused

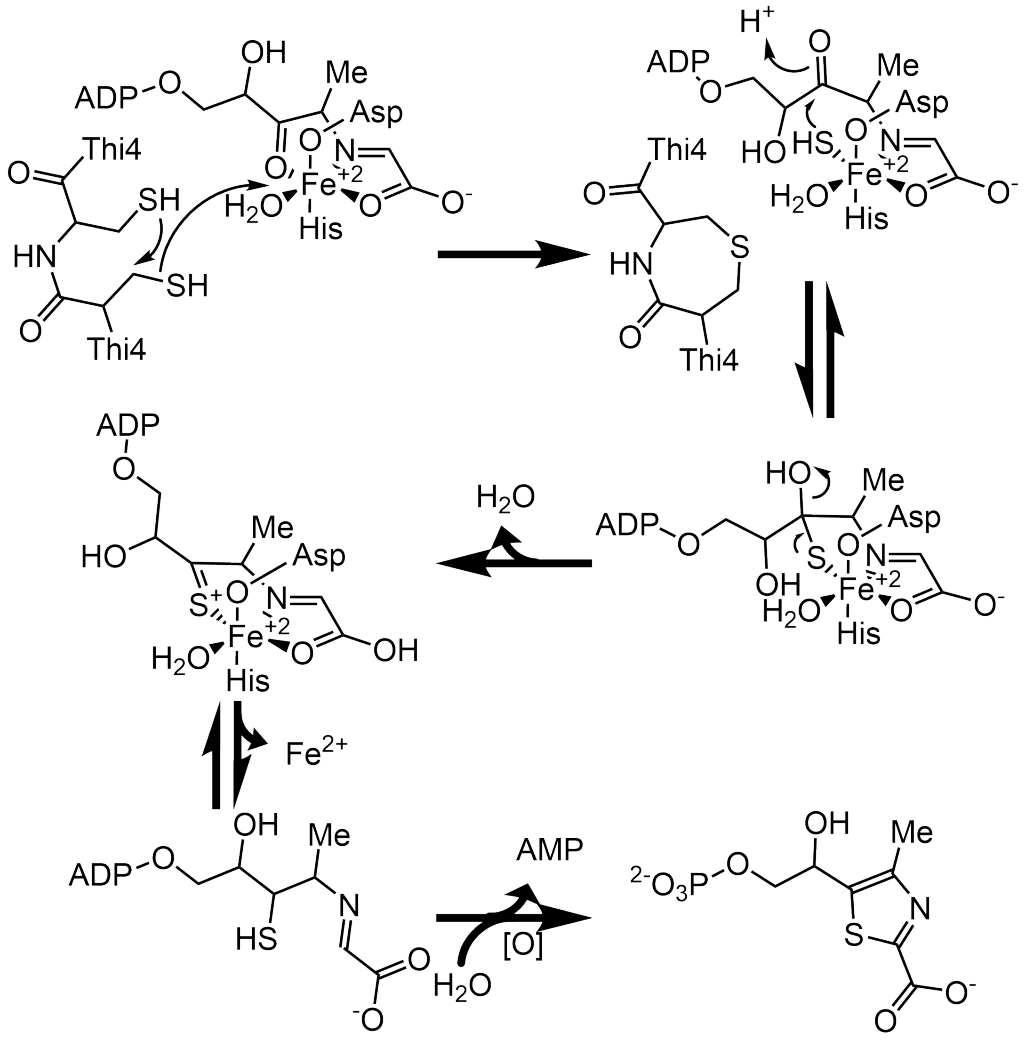

Figure 28: The iron dependent Thi4 reactions.

on the sulfur source for the enzyme and the role of iron in the reaction. The incorporated sulfur for most Thi4 homologs has been identified as Cys205 of the Thi4 protein, making it a suicide enzyme *in vitro*. The resulting dethiolated residue appears to form a thioether with the adjacent Cys204.[199,200] Whether this cysteine can then be regenerated, or Thi4 is a single use cosubstrate has yet to be conclusively demonstrated. Several archaeal organisms found in environments with high hydrogen sulfide concentrations appear to use ambient hydrogen sulfide as the thiol source and have replaced this otherwise vital Cys205 with a serine.[196,201] It

has also been found that *S. cerevisiae* C205S Thi4 mutants consume hydrogen sulfide and no longer acts like a suicide enzyme *in vitro*.[196]

The role of iron in Thi4 activity is another area of active research. The ion appears to be a transient feature in the enzyme structure, coordinated by the substrate, an aspartate, and a histone residue. Once coordinated it appears to activate the sulfide to allow it to attack the ketol group and attach to the substrate. It has been proposed that the iron then dissociates from the complex to allow the rest of the cyclization to proceed (Fig 28).[196,199,201]

4.4 Firefly Luciferin

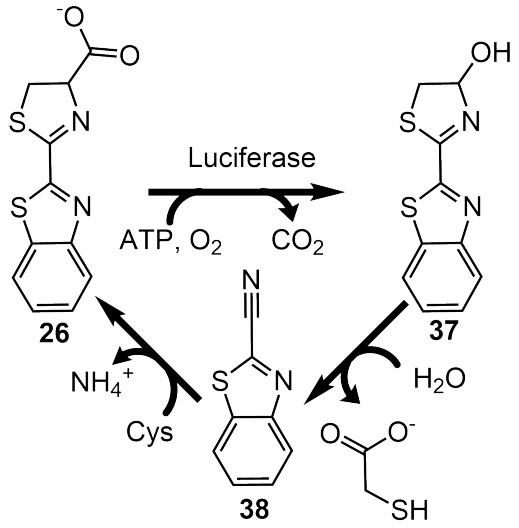

Figure 29: Light producing luciferin cycle.

Many a poet has written on the beauty of fireflies flying on a warm summer night. In addition to their atheistic value, firefly luciferase has also been a valuable tool for numerous biological assays. Fireflies are not the only organism to produce light, collectively the enzymes responsible for bioluminescence are referred to as luciferases, and the molecules they act on, luciferins.

Firefly luciferin **26** is the only one of these diverse molecules that contains a sulfur heterocycle, and therefore the only one that will be noted. Firefly luciferin

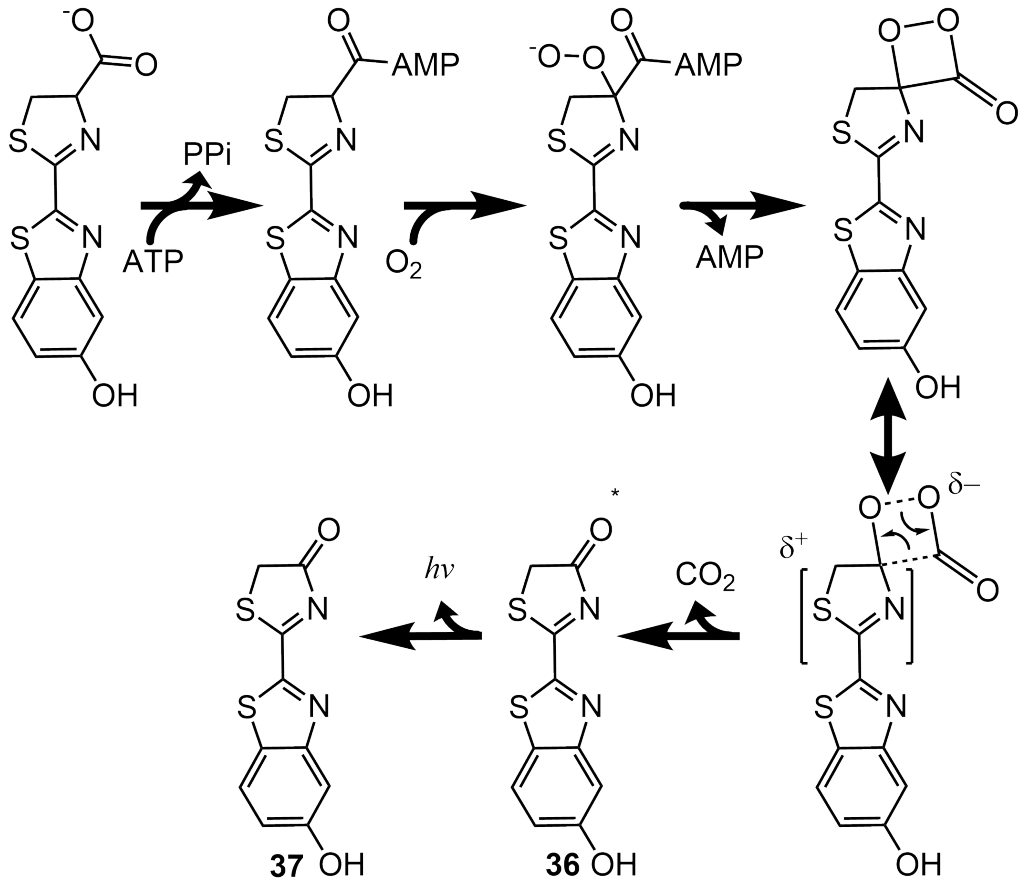

Figure 30: Luciferase catalyzed light producing reaction.

26 contains both an thiazole and a thiazoline moiety in its structure, but beyond data showing that the compound is constructed from two D-cysteins and a p-benzoquinone the biosynthetic pathway remains unknown.[202] The light emitting reaction involves the peroxidation of the thiazole through a carbanion intermediate (Fig 30). A single electron transfer mechanism to circumvent the spin forbidden process of binding the oxygen directly to the carbanion.[203] This results in a decarboxylation of the luciferin **26** and the gereration of an excited oxyluciferin **36** which rapidly decays to the ground state **37**, releasing a photon.[204] The thiazoline ring serves to initiate the breaking of the oxygen-oxygen bond by donating a partial negative charge to initiate the dissolution of the bond.[204] After the chemical's photon emitting reaction, the thiazoline is degraded and the sulfur

removed as part of thioglycolic acid (Fig 29). This moiety can then be regenerated by the nonenzymatic reaction of D-cysteine with the cyano group of 2-cyano-6-hydroxybenzothiazole **38**.[205] No work examining the possibility of an enzyme to accelerate this reaction have been published.

4.5 Understudied Thiazole Compounds

Despite the relative simplicity of the motif there are several thiazole and thiazoline compounds that have little more known about their biosynthesis than what organism produces them. Dercitin **30** is a compound isolated from *Dercitus* sea sponges that exhibits anti-tumor activity. It works by intercalating the DNA, similar to other pyridoacridine antibiotics.[206,207] S1319 **28** is a *Dysidea* sponge product. It is a β2-adrenoceptor agonist and has been shown to inhibit IgE mediated mast cell activation.[208,209] Violatinctamine **29** comes from the tunicate *Cystodytes cf. violatinctus*, and was identified in a comprehensive secondary metabolite screening effort.[210] One of the challenges of studying the biosynthesis these compounds is that they are produced by multicellular organisms, which are both more difficult and expensive to study than bacteria. To further complicate things, it is difficult to determine which secondary metabolites isolated from Porifera and Tunicate sources are actually produced by their normal flora. Microbes associated with these organisms are notoriously difficult to isolate in pure culture, inhibiting direct study of their metabolic pathways.[211,212]

5 Cephams and Other Six Member Heterocycles

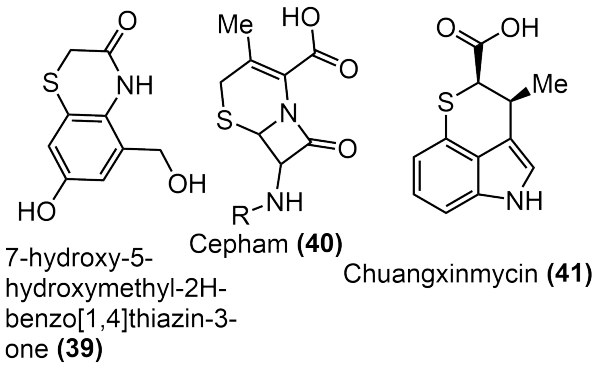

Figure 31: Metabolites with six member heterocycle rings.

5.1 Cephams

Like penams **25**, cephams **40** are beta lactam antibiotics with a fused biheterocyclic ring system, where the larger ring contains a sulfur. As may be predicted by their similarities, the two antibiotic families share the same early biosynthetic steps, branching into different pathways after IPNS makes the beta lactam ring (Fig 23). After IPNS, penam **25** antibiotics add diversity by modifying the side chains attached to the bicyclic core of IPN, cephams **40** by contrast expand the five member sulfur containing ring to a six member ring before diversifying the side chains (Fig 33).[213] The enzyme involved in this ring expansion is often referred to as penicillin N expandase, especially in the early literature. It is now more common to find it referred to as deacetoxycephalosporin C synthase (DAOCS) in the literature. The conversion of penicillin N to cephalosporin C is by far the most favored conversion for the enzyme, but in vitro DAOCS can also catalyze the ring expansion of other penams **25**, though at severely reduced efficiencies.[214] DAOCS, like IPNS, is an iron dependent non-heme monooxygenase, specifically an AKG dependent oxygenase. Unlike IPNS this enzyme is functionally, as well as structurally, a member of the AKG oxygenase

family, using two electrons from AKG to produce the iron IV oxo species necessary for activity.[215,216]

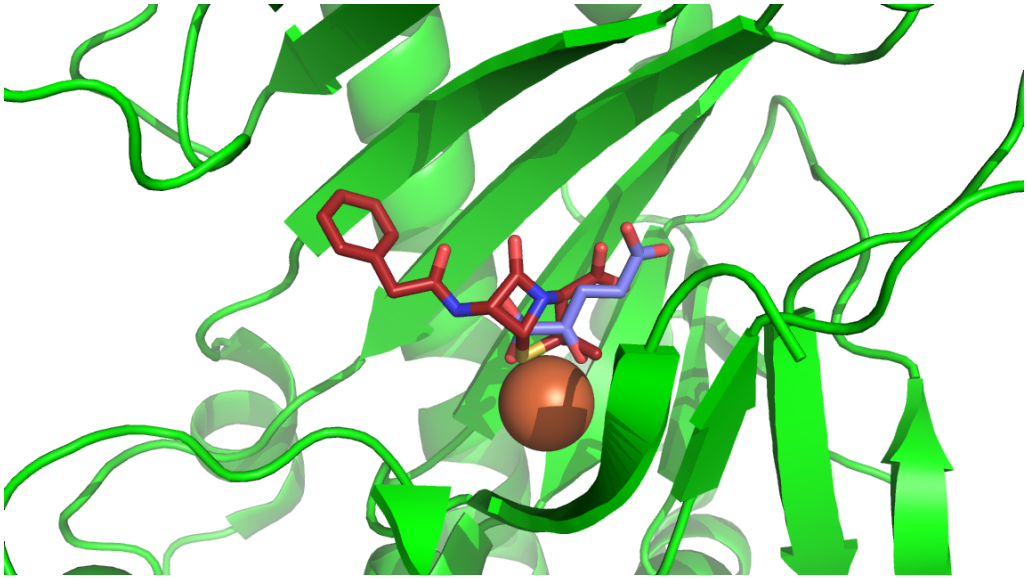

Figure 32: Crystal structure of DAOCS (green) with both penicillin G (dark red) and AKG (light blue).

There is some ambiguity as to whether this enzyme's mechanism is analogous to others of the same family. Crystallography of DAOCS bound to either penicillin N or AKG show the two substrates coordinating around the iron atom occupy the same space (Fig 32), suggesting that the AKG is released from the iron center after donating its two electrons.[217,218] Mass spectroscopy, NMR, and UV experiments using the soluble form of the enzyme support the canonical mechanism of AKG and penicillin N binding at the same time.[219] This would suggest that a crystallization artifact caused AKG, penicillin N, or both to bind improperly, however the crystals have been found to be catalytically active upon soaking with the substrate,[217] suggesting that any artifacts present are minor.

Regardless of whether penicillin N can bind concurrently with AKG, the vast majority of papers accept that the reaction occurs through what is referred to as a Morin rearrangement (Fig 33).[219] In this mechanism, the iron IV oxo substrate abstracts a hydrogen from the pro-R methyl on the five member ring, forming a

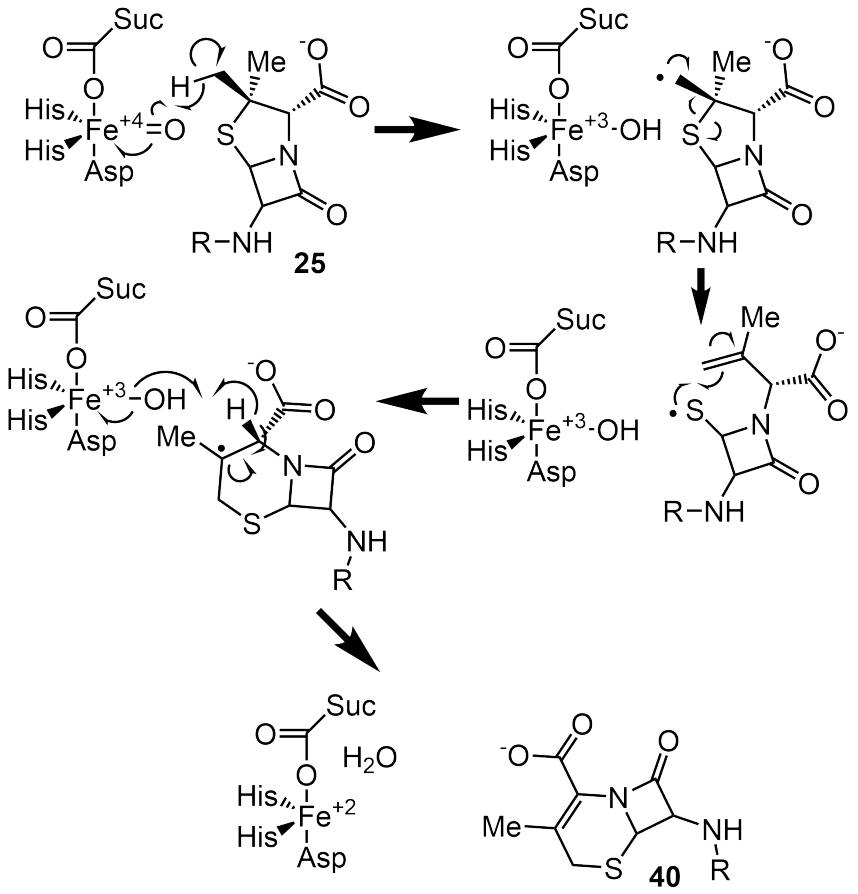

Figure 33: Cepham ring expansion mechanism.

primary carbon radical. This radical forms a double bond by breaking the carbon-sulfur bond of the larger ring. This electrophilic double bond then interacts with the nucleophilic thiol group to expand the ring into a hexameric cycle. This bond forms a tertiary carbocation that is resolved by the formation of a double bond accompanied by the abstraction of a hydrogen from the proximal carbon.[220]

A significant amount of work on DAOCS has been focused on developing enzymes capable of accepting penicillin G as a substrate in place of the more expensive and less stable penicillin N. The native enzyme has surprising specificity toward penicillin N, even over the IPNS product, isopenicillin, which only differs in the stereochemistry of the end of the α-aminoadipic acid moiety. Penicillin N has the R stereochemistry, while isopenicillin N is S. Thus far the most effective

modifications to improve penicillin G activity are localized in the C terminus, as would be expected from the crystal structure.[221,222]

5.2 Understudied Compounds

For an antibiotic that was discovered approximately four decades ago,[223] there is surprisingly little known about the biosynthesis of chuangxinmycin **41**. Recent discovery of similar compounds in the producing strain, *Actinoplanes tsinanensis*, may serve to shed light on the compound's biosynthesis, but at this time it is unclear whether such compounds are intermediates or variations of the antibiotic.[224] 7-Hydroxy-5-hydroxymethyl-2H-benzo[1,4]thiazin-3-one **39** by contrast was discovered relatively recently in *Ampelomyces*.[225] Future work on this antibiotic promises a better understanding of its production, and hopefully a more elegant name.

6 Cruciferous Phytoalexins

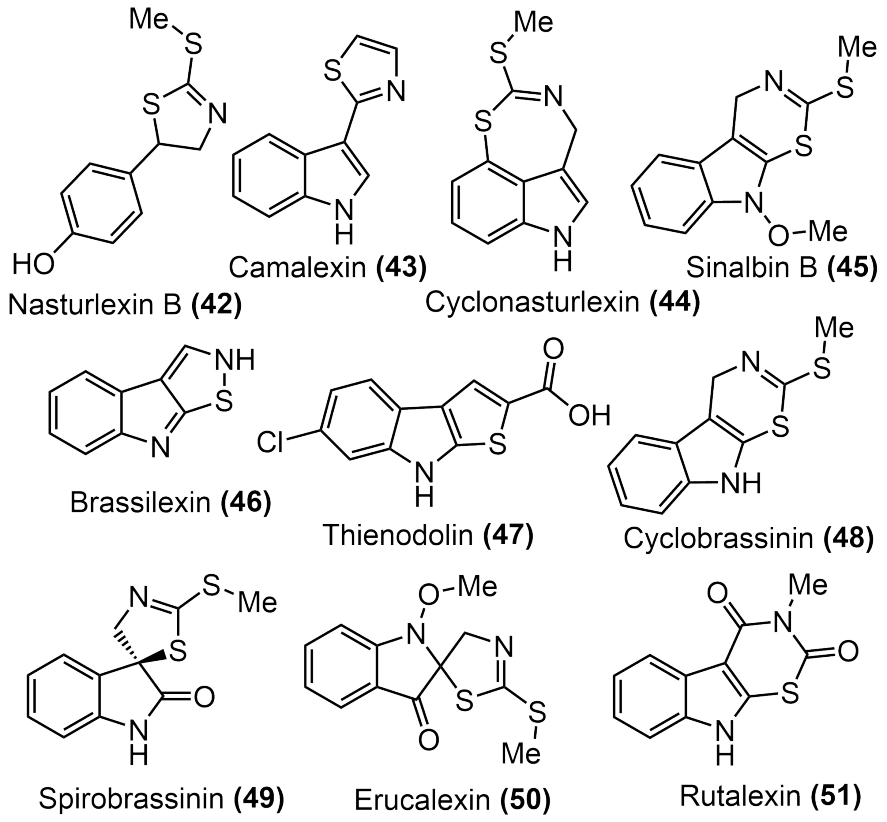

Figure 34: Cruciferous phytoalexins with sulfur containing heterocycles.

Phytoalexins are small molecules produced by plants in response to stress, generally bacterial or fungal attack, but also in response to the presence of heavy metals or an excess of UV radiation.[226,227] As would be expected for a class of compounds defined by their function there is a lot of molecular diversity within the classification, but within this diversity there are numerous groups which share structural similarities. Relevant to this review are the phytoalexins produced by the agriculturally important Brassicaceae family of plants, archaically known as Cruciferae.

Cruciferous phytoalexins predominantly consist of a tryptophan derived indole with a sulfur bearing moiety, often a heterocycle, bound at carbon three. There have recently been identified several phenylalanine derived cruciferous

phytoalexins, namely the nasturlexins **42**,[228,229] which also contain sulfur bearing heterocycles. Although a biosynthetic pathway for these phenylalanine derived heterocycles have been proposed, the lack of data to support this hypothesis makes it difficult to do more than mention them.

The diversity of these compounds and the conditions that stimulate their production suggests multiple mechanisms of action. Unfortunately the specifics of cruciferous phytoalexin activity have not been well studied.[230] Membrane disruption in bacteria is the most well established mode of action in examined compounds, but the mechanism of this disruption and the role of sulfur in the process is unknown.[231] Less relevant to the plant, but more relevant to medicine, several cruciferous phytoalexins have anti-proliferative affects. For camalexin **43** this anti-proliferative activity appears to be mediated by the generation of reactive oxygen species.[232]

As is often the case, the size and complexity of most plant genomes, and the lack of obvious organization, has made it difficult for researchers to determine the biosynthetic pathways of these compounds. Many of these phytoalexins appear to be derived from brassinin,[233–237] though how sulfur gets incorporated into brassinin has yet to be determined. It has been demonstrated that S-tryptophan **53** is a precursor of brassinin **52**,[238] and that the sulfur source is either cysteine directly, or a cysteine derived compound.[239] While there remains a great deal of work, recent papers have shed some light on the biosynthesis of camalexin **43**, theindolin **47**, spirobrassinin **49**, and cyclobrassinin **48**.

From brassinin **52** the P450 monooxygenases CYP71CR1 and CYP71CR2 can form spirobrassinin **49** and cyclobrassinin **48** respectively (Fig 35). The proposed mechanism for these two cyclizations is the formation of an epoxy ring with C2 and C3 of the indole, the following nucleophilic attack by sulfur forms the five or the six-member ring.[234] At least two variants, sinalbin B **45** and erucalexin **50**, are proposed to follow the same mechanism using a different precursor, namely

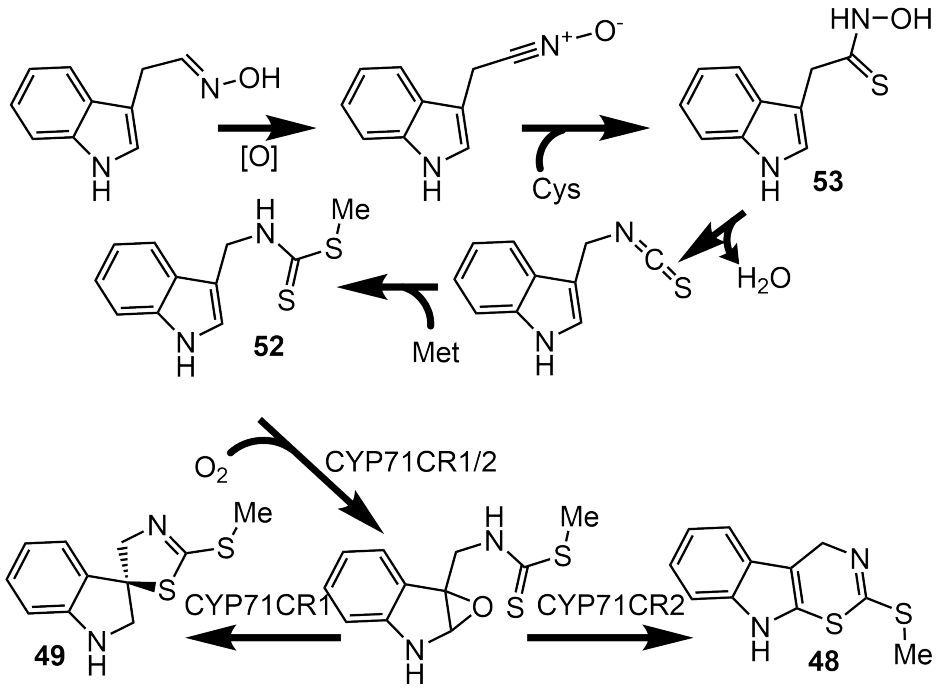

Figure 35: Proposed pathway for brassinin and derived products.

1-methoxybrassinin.[235] This theory cannot be confirmed however, until sinalbin B **45** and erucalexin's **50** biosynthetic enzymes are identified.

The minimal set of necessary enzymes for camalexin **43**, the predominant phytoalexin produced by *Arabidopsis thaliana*, have also been determined (Fig 36).[240,241] The initial steps of production from tryptophan requires two P450 cytochrome catalyzed oxidations, an oxidative decarboxylation performed by CYP79B3 or by CYP79B3 followed by a second oxidation by CYP71A13 to form indole-3-acetonitrile **54**.[240,242,243] The sulfur incorporation step that comes next appears to have several redundant mechanisms. The primary rout in vivo is the attachment of glutathiol by GSTF6 followed by the cleavage of the glutamate and glycine by GGP1 or GGP3 and carboxypeptidase respectively.[244,245] GH3.5 has also been found to be able to attach cysteine directly to indole-3-acetonitrile **54**, though with lower activity.[246] The attachment of the thiol is thought to be preceded by the activation of the target carbon by a hydroxyl, and it has been

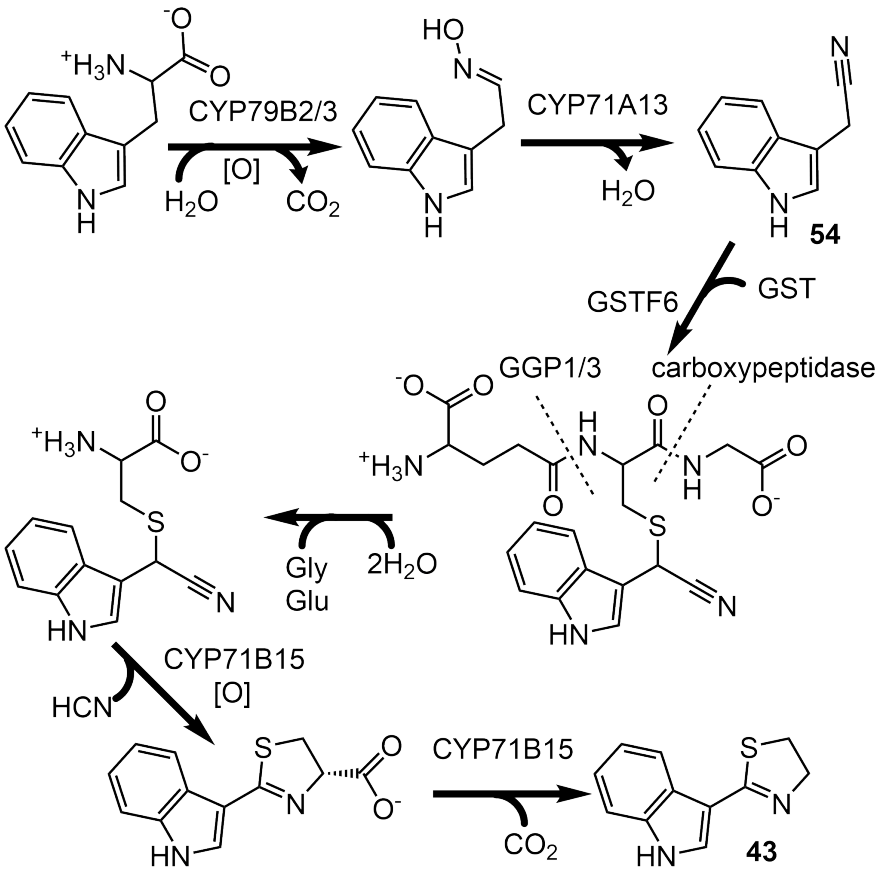

Figure 36: Proposed pathway for camalexin **43** biosynthesis.

found that CYP71A13 is able to hydroxylate the necessary site, though it is unclear if this activity is significant in vivo.[240] Regardless of how the cysteine is attached, the cyclization is catalyzed by CYP71B15, a third P450 that also catalyzes the final oxidative decarboxylation in camalexin **43** biosynthesis.[240,247] The chemistry for these proteins appears fairly conventional, so it is not terribly surprising that no work has been published examining the specific mechanisms of sulfur incorporation or cyclization.

The minimum set of enzymes involved in production of thienodolin **47** have been identified by heterologous expression in a non-producing strain, and a reasonable pathway has been proposed.[174] Unfortunately for this paper, the focus has been on halogen rather than sulfur incorporation or heterocyclization, and

these steps have yet to be examined in detail.

7 Thiosugars

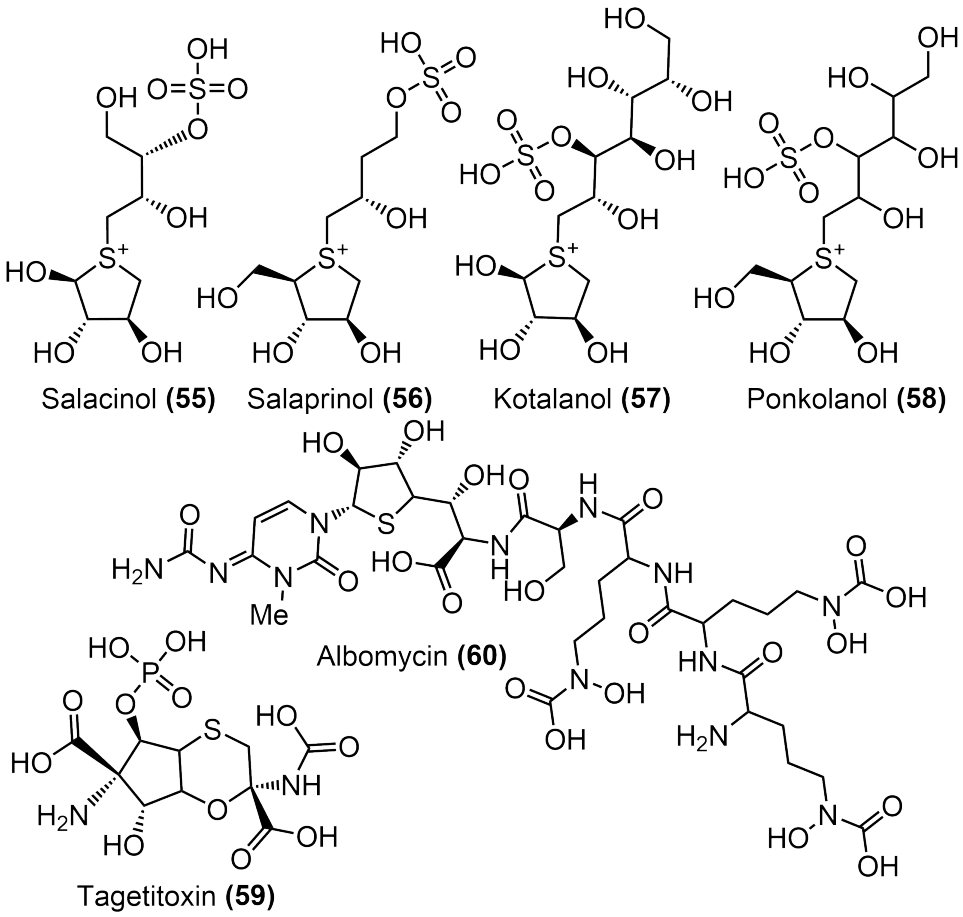

Figure 37: Heterocycle forming thiosugars.

Little is known about the incorporation of sulfur into thiosugars in general, much less those few thiosugars that form heterocycles. Kotalanol **57**, ponkolanol **58**, salacinol **55**, and salaprinol **56** are all extracted from *Salacia* speicies as part of Ayurvedic traditional medicine to treat diabetes.[248–251] Tagetitoxin **59** is an RNA polymerase inhibitor produced by *Pseudomonas syringae* that shows activity against bacteria, chloroplasts, and eukaryotes.[252] While the structure has been well established for all of these compounds, almost nothing is known about how they are produced in vivo.

Of all the heterocyclic thiosugars, the most research has been published on

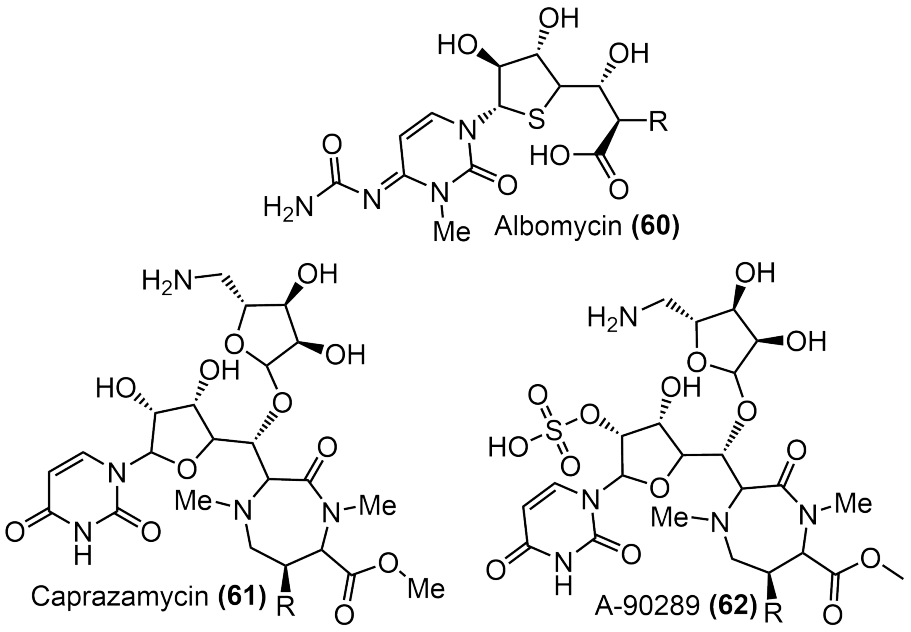

Figure 38: Sugar moieties with biosynthetic enzymes homologous to enzymes in the albomycin gene cluster.

the biosynthesis of albomycin **60**. This antibiotic uses a Trojan horse strategy in order to gain entrance to its target cell. The chelator moiety entices the cell to take it up into the cytoplasm, where the modified 4-thiofuranosyl cytosine moiety it cleaved off by native proteases.[253] The freed thiosugar can then inhibit seryl-tRNA synthases.[253,254] These functionally different moieties are also synthesized through two independent pathways before being joined by AbmR and AbmC. The chelator is formed from N^5-aceyl-N^5-hydroxyornithine by a fairly standard NRPS pathway, while much less is known about the thiosugars formation.[253] Inducing cysteine and homocysteine production in the cell also increases albomycin production, supporting the hypothesis that those compounds are at least indirectly incorporated into the thiosugar.[255] Based on homology to enzymes in the caprazamycin **61** and A-90289 **62** biosynthetic pathways, it has been proposed that AbmD, AbmF, AbmH, AbmJ, and AbmM are involved in the thiosugars formation.[253] However, neither caprazamycin **61** nor A-90289 **62** contain a thiosugar themselves, limiting the ability to propose reasonable detailed

pathways without further information.

8 Polythiol Rings

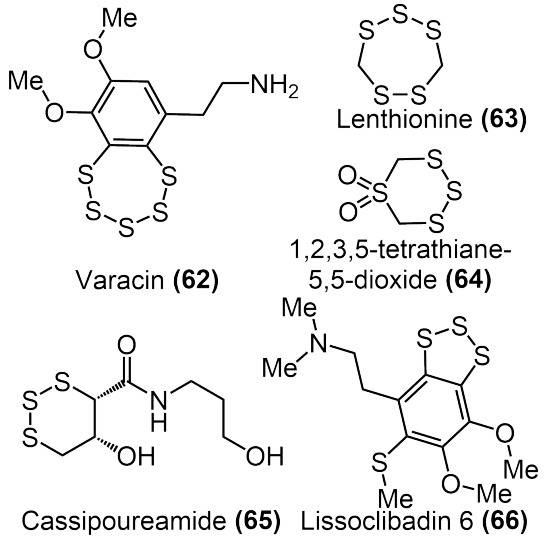

Figure 39: Heterocyclic products with more than two sulfurs.

For those natural products with more than two sulfurs in their heterocycles it is often difficult to differentiate intended products from isolation artifacts or minor side products, especially when there is so little data available on the compounds' biosynthesis. Indeed, lenthionine **63** is the only polysulfide heterocyclic metabolite with solid evidence to support direct enzymatic involvement. 1,2,3,5-Tetrathiane-5,5-dioxide **64** was identified from a screen of the red alga *Chondria californica*'s products.[256] Varacin **62** was isolated from *Polycitor* species and *Lissilinum tunicates*, and shows thiol mediated cleavage of DNA.[257,258] Several cytotoxic lissoclibadin **66** compounds have been isolated from *Lissoclinum cf. badium*, some of which contain sulfur heterocycles, and some of which do not.[259,260] Cassipoureamide **65** is found in *Cassipourea guianensis* wood and appears to have an insecticidal function.[261]

Lenthionine **63** is produced by the fruiting bodies of the *Lentinula edodes* fungus, better known as shiitake mushrooms, as well as the marine bacteria *Roseobacter denitrificans*.[262] While this compound has modest antibiotic activity, it

is far better known for the aroma and flavor it imparts on the edible mushroom.[263] Despite being isolated in 1966[264] there remain numerous questions about the synthesis of the compound, even to what extent its formation is enzymatic.[263] It has been established in the 1960s and 1970s that lenthionine's **63** formation from its precursor, lentinic acid,[265] requires at least a cysteine desulfurase[266] and a glutamyl transpeptidase[267] from the producing mushroom. From this point research as to lenthionines **63** biosynthesis has been for the most part stagnant. A recently identified but poorly characterized cysteine desulfurase, Lecsl, encoded by *L. edodes* may play a role in lenthionine biosynthesis. Phylogenetically, this PLP dependent enzyme fits poorly with previously identified cysteine desulfurases, forming its own third clade of cysteine desulfurases. Functionally, it is unusual since it is more efficient in cleaving the C-S bond in S-methyl-L-cysteine sulfoxide than in L-cysteine.[268] While the Lecsls role in lenthionine production has not been directly examined yet, it is consistent with the enzyme that was demonstrated to be involved in lenthionine production in 1971.[266,268]

9 Miscellaneous Heterocycles

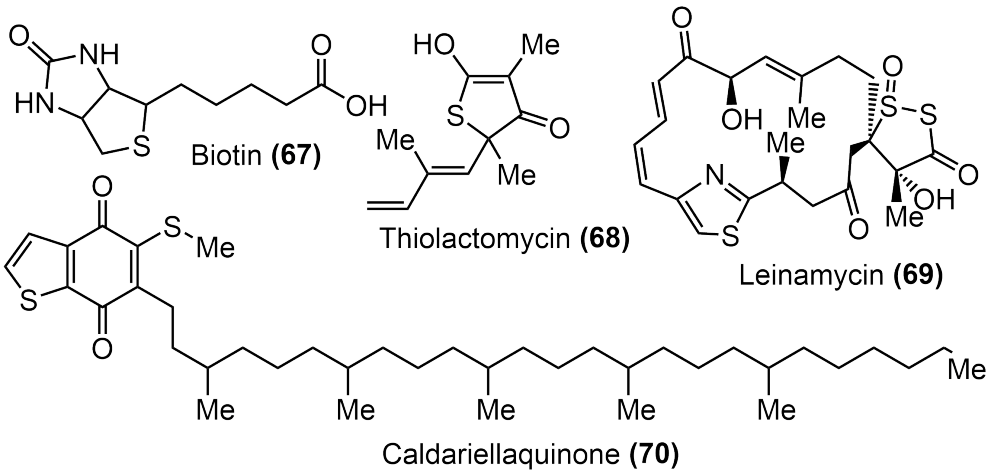

Figure 40: Heterocyclic products that do not fit into any of the previous sections.

9.1 Caldariellaquinone

Caldariellaquinone **70** appears to be an unusual electron carrier found in the acidothermophilic genus *Sulfolobus*. The compound was first identified in 1977. It has been confirmed that the electron carrying head group is derived from tyrosine, and that the tail is constructed from isoprene monomers.[269–271] How the sulfur is attached or how the pentameric heterocycle is closed remains a mystery.

9.2 Leinamycin

Leinamycin **69** is a PKS/NRPS derived broad spectrum antibiotic produced by *Streptomyces atroolivaceus*. The compound contains two heterocycles, a thiazole whose formation is similar to what has been previously discussed, and a 1,3-dioxo-1,2-dithiolane moiety which is significant for its anti-tumor and broad spectrum antibacterial activity.[272,273] The disulfide bond in the molecule can react with O_2 to generate reactive oxygen species and the reduced thiols can covalently modify guanine in the DNA.[274]

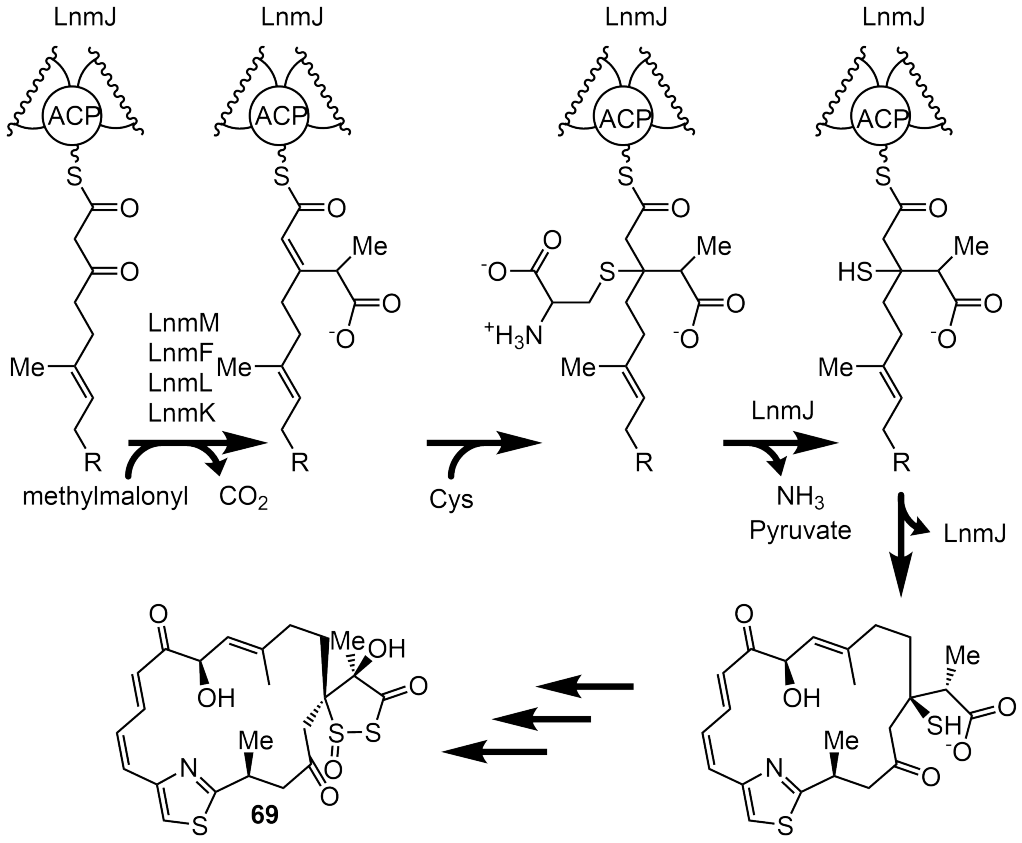

Figure 41: Sulfur incorporation into leinamycin.

The formation of this disulfide containing ring is an area of active research (Fig 41). While many of the components of the five-member ring appear to be added while the compound is bound to the final PKS complex, only one carbon of the five-member ring comes from the canonical PKS chain extension. The other three carbons are derived from a methylmalonyl moiety whose attachment and decarboxylation are catalyzed by the joint activity of LnmL, LnmK, LnmM, and LnmF.[275] The more proximal sulfur is derived from cysteine. It is most likely attached to the polyketide product by an as of yet uncharacterized domain of the LnmJ PKS domain. The attached cysteine is then cleaved by the recently characterized cysteine lyase domain of the same LmJ. This domain has been confirmed to break the C-S bond of cysteine in a PLP dependent manner without much specificity toward what else is attached to the sulfur.[272] Unfortunately, the

process to attach the second sulfur is currently unknown, as are the proteins which oxygenate and oxidize the disulfide bond.

9.3 Biotin

Biotin **67** is an essential cofactor involved in carboxylation within every domain of life (Fig 42). The catalytically important moiety consists of a ureido ring fused to a sulfur containing thiophane ring. This ureido moiety is covalently attached to enzymes by the activity of BirA through a fatty acid chain linked to the five member heterocycle. Crystal studies have shown that in the protein the urido sulfur interacts with lysine and/or the aeromatic ring of tyrosine,[276,277] possibly serving to better align the bicyclic ring. Outside of the enzyme, the sulfur component of biotin has been shown to decrease the pK_a of both urido nitrogens when compared to O-heterobiotin, facilitating the cofactor's carboxylation.[278]

Despite its necessity, humans and other animals have lost the ability to produce biotin **67**, and depend on intake from their diet and from biotin producing organisms in the gut to fulfill their required intake of the cofactor. The high prevalence of biotin **67** in most foods, the ease of recycling biotin **67** cofactor, and the exceedingly small quantities required clearly did not provide much evolutionary pressures for animals to maintain a biotin producing pathway, especially considering the unusually high energy cost. Biotin's **67** biosynthetic pathway contains two unusually high energy steps. First, a transaminase reaction catalyzed by BioA uses SAM as a nitrogen source instead of glutamate or some other amino acid. This reaction isn't directly expensive for the cell, but the deaminated SAM spontaneously degrades, requiring the cell to spend energy replenishing its supply. The second unusually energetically demanding step is in forming the ureido ring using an iron sulfur cluster as the sulfur source.[279]

The enzyme responsible for the last step of biotin **67** biosynthesis is the homodimeric protein BioB, also referred to as Biotin Synthase. Within it, two

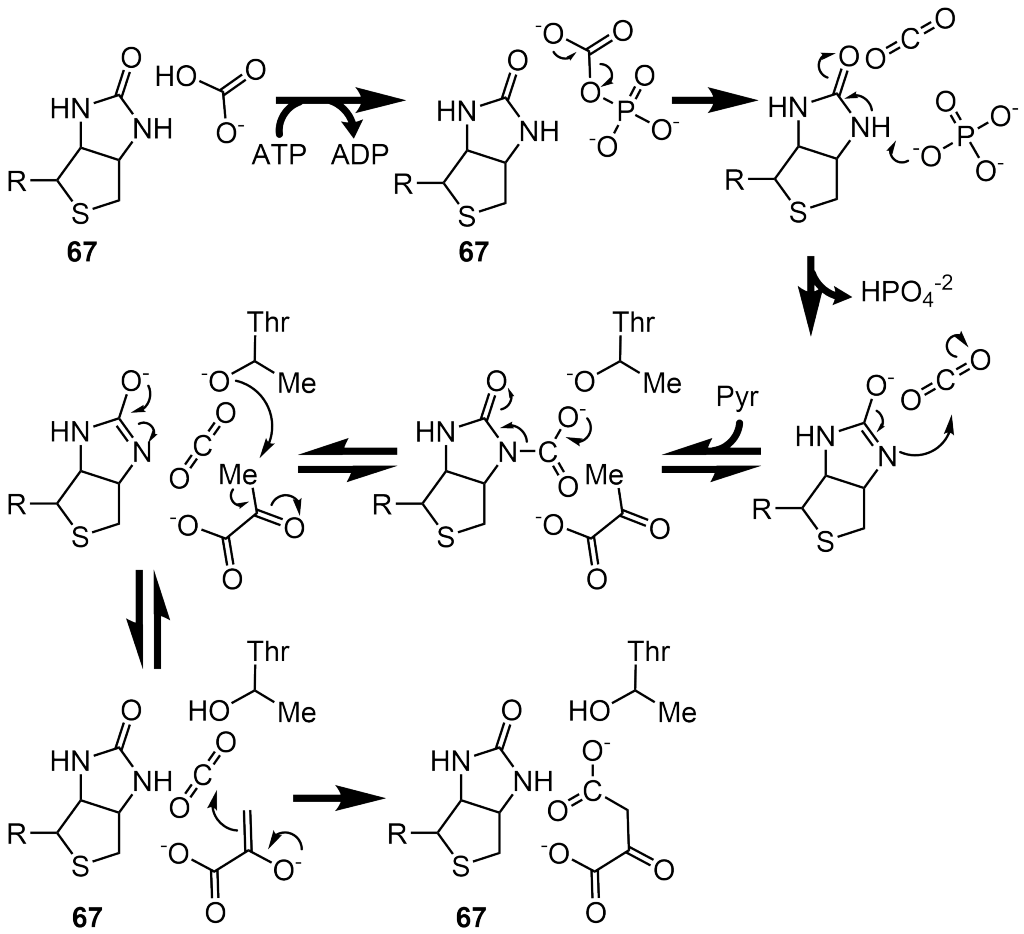

Figure 42: Pyruvate carboxylase mechanism.

molar equivalents of SAM are converted to Ado and methionine and one of its two iron sulfur clusters are degraded to produce a molar equivalent of biotin **67** (Fig 43). The first SAM is oxidized by a [4Fe-4S] cluster, generating dAdo•.[280–282] This radical abstracts a hydrogen from C9 of dethiobiotin (DTB) **71**, allowing it to attack a $[2Fe\text{-}2S]^{2+}$ cluster, covalently bonding to the μ sulfur.[280–285] This 9-mercapto DTB **72** is then held in the active site while dAdo and methionine are exchanged for a new SAM and the $[4Fe\text{-}4S]^{2+}$ cluster is reduced by a ferredoxin reductase back to $[4Fe\text{-}4S]^{+}$.[279,286,287] The second dAdo• abstracts a hydrogen from C6, causing it to attack the same μ sulfur and closing the ureido ring to complete biotin synthesis.[285]

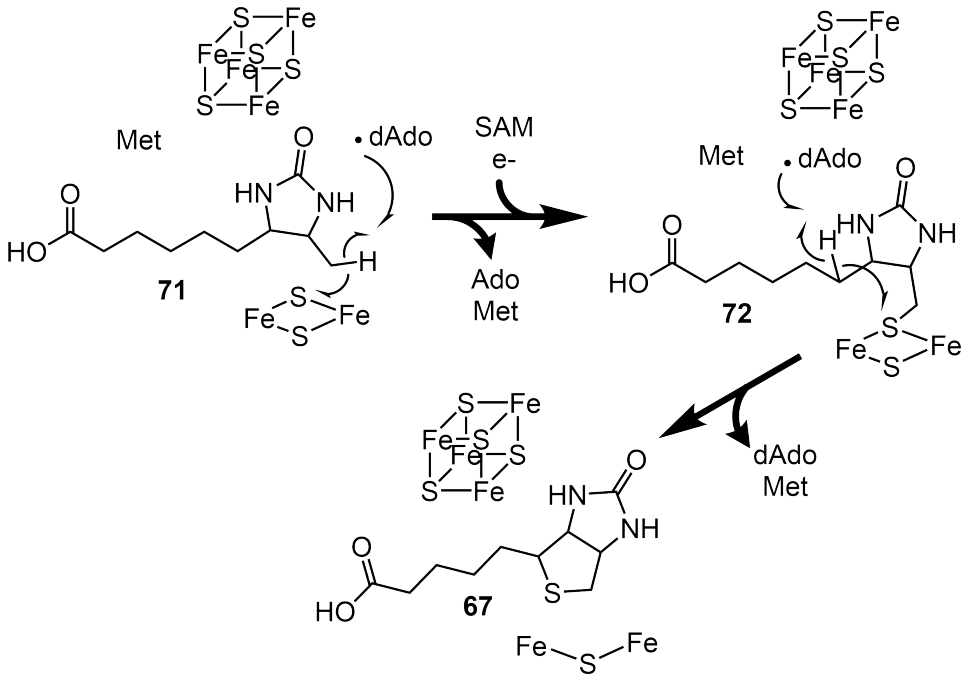

Figure 43: The BioB catalyzed reaction.

BioB is an unusual enzyme due to its two iron sulfur clusters and their very different roles in catalysis. The $[4Fe\text{-}4S]^{2+}$ is held by Cys53, Cys57, and Cys60 in a canonical CXXCXXXC motif. The positioning of the $[4Fe\text{-}4S]^{2+}$ cluster near the surface of the protein at the end of the beta barrel probably makes it easier for ferredoxin reductase to reduce the cofactor for reactivation.[281] The $[2Fe\text{-}2S]^{2+}$ cluster is coordinated by Cys97, Cys128, Cys188, and notably Arg260.[281] The three cysteines are well conserved, with serine or aspartate replacing Cys97, or alanine replacing Cys128 in a minority of examined genomes. The deprotonated Arg260[288] is a particularly curious residue because it is perfectly conserved in all of the examined sequences, but its importance to the protein's activity remains a mystery. The residue can be mutated to alanine, cysteine, histidine, and methionine without destroying the enzymes activity, making it difficult to determine why this position is so tightly conserved.[286,289]

Outside of the iron sulfur clusters, Asn151, Asn153, and Asn222 have been identified as forming hydrogen bonds with the substrate. Asn153 appears to be

particularly important in binding to the 9-mercapto DTB **72** intermediate, as mutants at this position allow the intermediate to dissociate from the protein before the second SAM can abstract the hydrogen to close the cycle.[287,290] Asn153 and Asp155 have been identified as important in binding and coordinating SAM.[280,290]

The mechanism by which the [2Fe-2S] cluster is regenerated after removal of the sulfur has yet to be definitively determined. The most likely pathway at the moment is that the ATP dependent chaperone HscA unfolds the protein so that the cluster can be replaced by other proteins in the ISC system. It has been demonstrated that HscA increases the number of BioB reaction turnovers, and binds more tightly to the [2Fe-2S] cluster depleted form of the protein than to the holoprotein.[291] This would appear to be a very energetically inefficient way to produce biotin.**67** Although IscU mutants have demonstrated that a redundant iron sulfur cluster regenerating pathway exists to regenerate BioB,[291] no mechanism of BioB regeneration has experimental support. Considering the very small quantities of biotin **67** required by the cell, it may be more energy efficient to completely unfold the protein with the ISC system than it would be to maintain an additional protein to specifically regenerate BioB.

9.4 Thiolactomycin

Thiolactomycin **68** is a PKS derived product produced by members of the *Nocardia* and *Streptomyces* genus that inhibits type II fatty acid synthases by binding the malonyl-ACP pocket in FabB.[292,293] Despite its structure being determined in 1989[294] and strong evidence published in 2003 of it being produced by a PKS,[292] no gene cluster was identified until 2015.[295] The core of the product is produced as would be expected from an acetate and three methylmalonates, however the proposed mechanism of sulfur incorporation and cyclization, if correct, is an unusual process (fig 44).

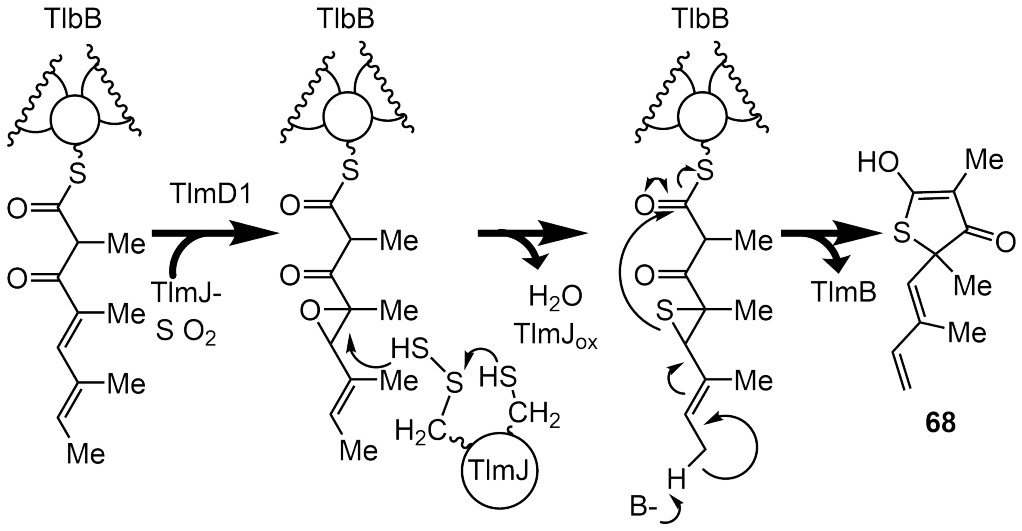

Figure 44: Hypothetical thiolactomycin cyclization.

Radiolabeling has demonstrated that cysteine is the sulfur source.[292] This is supported by the presence of an NRPS component of TlmB which appears to accept cysteine even though all carbons are accounted for from the PKS components of the pathway. These NRPS modules probably allow for TlmS, a cysteine desulfurase, to transfer the thiol group to TlmH, which has homology with tRNA-specific 2-thiouridylases. Activation of the thiolation site is proposed to occur by the formation of an epoxide rind by the P450 TlmD1. Knock out experiments determined that TlmD1 is required for product formation even though tall oxygens or oxidations in the core structure are accounted for by the PKS modules. It has been proposed that the epoxide ring is replaced by a thiirane ring by two subsequent nucleophilic attacks. The sulfur may then perform one final intramolecular electrophilic attack to convert this thiirane ring to the ultimate five-member heterocycle.[295]

10 Closing Remarks

We are entering the era of big data when it comes to biology. Next generation sequencing and high throughput systems give us more information than any human being could possibly comprehend. Global databases of research do not just report findings, they are rich sources of research themselves as scientists search through their content for patterns that further our understanding of biology without growing a single cell. With so much information at our fingertips it is easy to forget how much *in vitro* and *in vivo* work remains to be done. This review has shown how identical chemical moieties can be produced by very different chemical pathways, as well as how similar chemical structures can serve very different purposes in biology. More research is necessary on these understudied moieties before the true power of computational biology can be brought to bear on these pathways. Not just for the academic value of knowing how biology works, but to expand the tool kit available to use that biology for our collective benefit.

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